Fatty acids in human evolution

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Fatty acids in human evolution:
contributions to evolutionary medicine

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Om weer helder te kunnen zien hoef je vaak alleen van perspectief te veranderen.
Antoine de Saint-Exupéry

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List of abbreviations:

AA	arachidonic acid (20:4ω6)
AFP	alpha-fetoprotein
AGA	appropriate for gestational age infant
AT	adipose tissue
ACC	acetyl-Coenzyme A carboxylase
ALA	α-linolenic acid (18:3ω3)
Au.	*Australopithecus*
Ar.	*Ardipithecus*
BAT	brown adipose tissue
BHT	butylhydroxytoluene
BMI	body mass index
BP	before present
C_3/C_4	C_3- and C_4-photosynthetic pathway
ChREBP	carbohydrate responsive element binding protein
CAD	coronary artery disease
CHO	carbohydrates
CRL	crown-rump length
CVD	cardiovascular disease
DEE	daily energy expenditure
DHA	docosahexaenoic acid (22:6ω3)
DNA	deoxyribonucleic acid (the genome)
DNL	*de novo* lipid synthesis or *de novo* lipogenesis
DQ	dietary quality
DS	disaccharides
EBM	evidence based medicine
EBN	evidence based nutrition
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acids
EFAD	essential fatty acid deficiency
Elovl-6	elongation of very long chained fatty acids family member 6
EN%	energy %
EPA	eicosapentaenoic acid (20:5ω3)
EQ	encephalization quotient
FA	fatty acid(s)
FADS	fatty acid desaturase
FAME	fatty acid methyl esters
FAS	fatty acid synthase
FFA	free fatty acids
FH	fundal height
GI	glycemic index
GDM	gestational diabetes mellitus
H.	*Homo*
HC	head circumference
HCl	hydrochloric acid
HDL	high density lipoprotein
ISSFAL	International Society for the Study of Fatty Acids and Lipids
KY(A)	thousand years (ago)
LA	linoleic acid (18:2ω6)
LCPUFA (short: LCP)	long-chain (≥3 double bonds, ≥ 20 C-atoms) polyunsaturated fatty acids
LCSAFA (short: LCFA)	long chain (≥ 16 C-atoms) saturated fatty acids
LDL	low density lipoprotein
LGA	large for gestational age

LMP	last menstrual period
LPL	lipoprotein lipase
LXR	liver-X-receptor
LT	leukotrienes
LTB_4	leukotriene-B_4
MCSAFA (short: MCFA)	medium chain (≤ 14 C-atoms) saturated fatty acids
MS	monosaccharides
MUFA	monounsaturated fatty acids
MY(A)	million years (ago)
NAFLD	non-alcoholic fatty liver disease
NIMR	National Institute for Medical Research in Tanzania
NFkB	nuclear factor kappa B
OMP	observed movement patterns
P.	*Paranthropus*
PAL	physical activity level
PC	phosphatidyl-choline
PE	phosphatidyl-ethanolamine
PE	preeclampsia
PIH	pregnancy induced hypertension
PS	phosphatidyl-serine
PG	prostaglandins
PGI_2	prostaglandin I_2
PL	phospholipid
PPAR	peroxisome proliferator receptors
PP	postpartum
PS	polysaccharides
PUFA	polyunsaturated fatty acids (≥2 double bonds, ≥ 18 C-atoms)
RAR	retinoic acid receptors
RBC	red blood cell or erythrocyte
RCT	randomized controlled trial
RMR	resting metabolic rate
RXR	retinoid X receptors
PBB	Pee Bee Belemnite
SAFA	saturated fatty acids
SCD-1	stearoyl-Coenzyme A desaturase family member 1
Sp.	species
SREBP-1	sterol regulatory element binding protein-1
T1DM	type 1 diabetes mellitus
T(A)G	triacylglycerides
TC	total cholesterol
TEE	total energy expenditure
TR	thyroid hormone receptors
TNF	tumor necrosis factor
Tx	tromboxane
TxA_2	tromboxane A_2
UA	umbilical artery
UV	umbilical vein
VDR	the vitamin D receptor
VLDL	very low density lipoprotein
WAT	white adipose tissue
WHO	World Health Organization

Scope of the thesis

SCOPE OF THE THESIS

The studies in this thesis aim at the gathering of information on our ancient diet. This information is important to both public health and health care. We believe that its incorporation in nutritional science will be of crucial importance to achieve an increase of our number of years in health, by some referred to as 'healthy aging'.

To gather information on the composition of our ancient diet, we reconstructed, by theoretical means, the macronutrient and fatty acid (FA) compositions of the diets consumed by our ancestors living in the East-African land-water ecosystems. Secondly, we studied traditionally living people in East-Africa and compared their nutritional status with that of people living in Western countries. In the various studies we focussed notably on FA and especially arachidonic (AA) and docosahexaenoic acid (DHA), since the current evidence supports that both these long-chain polyunsaturated (LCP) FA were abundantly present from our diets in the past. Both, the reconstructed dietary composition and the dietary composition of some of the traditionally living East African people may serve as platforms for future intervention studies using the currently reigning paradigm of "Evidence Based Medicine", that is referred to as "Evidence Based Nutrition" in nutritional sciences.

Chapter 1 provides the background of the concept of Evolutionary Medicine, its common arguments and counterarguments and its implications. Secondly, the available evidence for the ecological niche, and hence the diet, of our early ancestors is reviewed. Finally, we discuss the intervention trials that have thus far been conducted with diets that mimic our Paleolithic nutrition.

Chapter 2 describes the composition of the foods available in the East-African land-water ecosystem. From both laboratory analyses and the available information in the literature, we reconstructed the macronutrient and FA compositions of the diets that could have been consumed by our earliest ancestors living in the East-African cradle of mankind.

Chapter 3 contains several studies on the FA composition of human milk in traditionally eating East-African populations. The locations of research are shown in **Figure 1**, the characteristics of the project and the project subjects are in **Figure 2-11**. Chapter 3.1 describes the FA composition of the milk of women living in lake-shore communities in the mainland of Tanzania, where freshwater fish is the main source of protein. In Chapter 3.2 we describe the milk FA compositions of several Tanzanian tribes with markedly different dietary habits. The outcomes are compared with the recommendations that have been issued by Western Nutritional Boards. Chapter 3.3 provides a discussion on the discrepancy between the current recommendation for the composition of infant formulas and the recommendation for LCP intakes by adults (i.e. 2 times/week of which 1 time fatty fish; or 450 mg eicosapentaenoic acid (EPA) + DHA/day). The current recommendation for infant

Figure 1. The different locations of research throughout Tanzania. From North to South: 1. Ukerewe island, home to the WaKerewe; 2. Sengerema, home to the WaSukuma and WaZinza; 3. Mwanza, home to the WaSukuma; 4. Wasso, home to the Maasai; 5. Digodigo, home to the WaSonjo; 6. Yaeda Chini, home to the Hadzabe (WaTindiga); 7. Haidom, home to the WaIraqw and the WaBarabaig (WaDatoga); 8. Kiomboi and Lake Kitangiri, home to the WaNyiramba; 9. Same, home to the WaPare and WaSambaa; 10. Ruvu on the Maasai Plains, home to the Maasai; 11. Dar es Salaam, mixed Afro-Arab peoples; 12. Chole island, Mafia Archipelago, mixed Afro-Arab peoples; 13. Matema beach, Lake Malawi, home to the WaNyakius.

formulas derives from the average milk composition of women who do not comply with the 450 mg EPA+DHA/day recommendation. Chapter 3.4 describes the FA differences between preterm and term milks from an African population with high intakes of LCP from freshwater fish. The possible causes for the observed differences are discussed.

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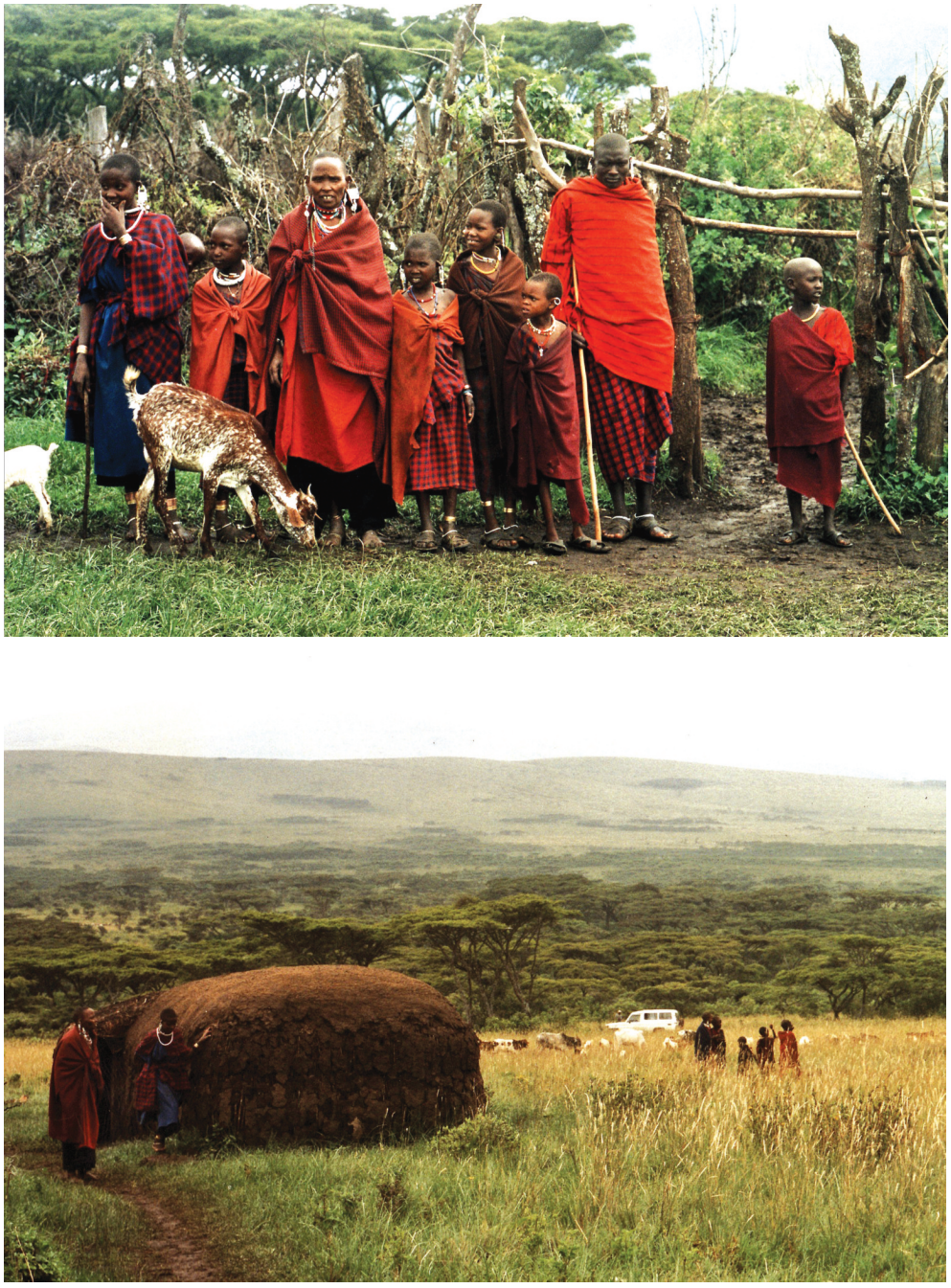

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Chapter 5 provides a description of the FA compositions of brain, liver and adipose tissue from African infants who were stillborn to women with very high intakes of freshwater fish. The FA-compositions of these tissues are compared with those of counterpart Western infants born to mothers with relatively low fish intakes.

Chapter 6 describes the development of the adipose tissue compartment in a migrant African population living in the former Netherlands Antilles. Chapter 6.1 presents the FA composition of fetal adipose tissue at several stages during gestation. In Chapter 6.2 the whole fetal body LCP content is reconstructed from the available data from our previous studies and from the literature. From these data, the daily FA increment rates in the fetal body during gestation were calculated. These data might be useful for the compilation of infant formulas.

Chapter 7 focuses on the mechanisms that might be at the basis of the observed differences in FA status between certain cases and controls and on the observed changes in FA status with advancing gestation and after delivery. Chapter 7.1 provides the differences in umbilical vessel wall FA status between women after preeclamptic and uncomplicated gestation. The observed differences are related to differences in glucose and insulin metabolism. Chapter 7.2 elaborates on the possible influences of insulin sensitivity and DHA status on the FA composition of maternal and infant RBC during pregnancy and subsequent lactation. Chapter 7.3 discusses the possible influences of insulin sensitivity on the observed differences in the milk FA composition between preterm and term milks and the changes with advancing lactation.

Chapter 8 provides a review of the available evidence on the roles of carbohydrates (CHO) and saturated fatty acids (SAFA) in cardiovascular disease (CVD).

In **Chapter 9** we summarize the main conclusions and we discuss the limitations and the implications of our findings.

CHAPTER 1

General introduction

A multidisciplinary reconstruction of Paleolithic nutrition that holds promise for prevention and treatment of diseases of civilization

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ABSTRACT

Evolutionary medicine acknowledges that many chronic degenerative diseases result from conflicts between our rapidly changing environment, our dietary habits included, and our genome, which has remained virtually unchanged since the Palaeolithic. Reconstruction of the diet prior to the agricultural and industrial revolutions is therefore indicated, but hampered by the ongoing debate on our ancestors' ecological niche. Arguments and their counterarguments regarding evolutionary medicine are updated and the evidence for the long-reigning hypothesis of human evolution on the arid savannah is weighed against the hypothesis that they evolved in the proximity of water. Evidence from various disciplines is discussed, including the study of palaeoenvironments, comparative anatomy, biogeochemistry, archaeology, anthropology, (patho)physiology and epidemiology. Although our ancestors had much lower life expectancies, the current evidence does neither support the misconception that during the Palaeolithic there were no elderly nor that they had poor health. Rather than rejecting the possibility of 'healthy ageing', the default assumption should be that healthy ageing posed an evolutionary advantage for human survival. There is ample evidence that our ancestors lived in a land-water ecosystem and extracted a substantial part of their diets from both terrestrial and aquatic resources. Rather than rejecting this possibility by lack of evidence, the default assumption should be that *hominins*, living in coastal ecosystems with catchable aquatic resources, consumed these resources. Finally, the composition and merits of so-called 'Palaeolithic diets,' based on different hominin niche-reconstructions, are evaluated. The benefits of these diets illustrate that it is time to incorporate this knowledge into dietary recommendations.

INTRODUCTION

In the Origin of Species,[1] Darwin recognized that there are two forces of evolution, i.e. *natural selection* and *the conditions of existence*, where the latter was considered the most powerful.[2] For example, important steps in evolution are the origin of eukaryotic life approximately 1.6-2.7 billion years ago[3,4] and the appearance of photosynthetic cyanobacteria that began to oxygenate the atmosphere around 2,400 million years ago.[5] However, there was relatively little alteration in the design of life forms before the Cambrian explosion about 600 million years ago. Only when the oxygen tension in the atmosphere rose above the Pasteur point, aerobic metabolism became thermodynamically possible[6] resulting in an explosion from simple prokaryotics to a diversity of eukaryotic life forms.[7]

During the past millions of years of evolution, with relatively little alteration in life forms and environmental circumstances, the human genome has become optimally adapted to its local environment.[8-11] In other words, our genome may have reached a state of homeostasis, defined as the 'optimal interaction between environment and genome' or 'nature in balance with nurture', to support optimal survival for reproductive success. The etiologies of many typically Western diseases, also known as diseases of affluence or civilization, have been attributed to the disturbance of this delicate balance, secondary to the rapid changes in the conditions of existence, while our genome has remained basically unchanged since the beginning of the Palaeolithic era. The former include changes in physical activity, stress, sleep duration, environmental pollution and others,[12,13] but one of the most rapidly changing conditions of existence has been the human diet.

Since the onset of the agricultural revolution, some 10.000 years ago, and notably in the last 200 years following the start of the industrial revolution, humans have markedly changed their dietary habits. Consequently, it has been advocated that the current pandemic of diseases of civilization result in part from the mismatch between the current diet and our Palaeolithic genome. In other words, "we are what we eat, but we should be what we ate".[14,15] The ensuing poorly-adapted phenotype may find its origin as early as in the fetal period[16,17] and possibly as far back as in the maternal grandmother's womb.[18] This phenotype might be laid down in, inherently labile, epigenetic marks that are meant for the short and intermediate term adaptation of a phenotype to the conditions of existence. With clear evolutionary advantages they may become transmitted to the next generations as a memory of the environmental conditions that can be expected after birth.[19] They thereby give rise to a seemingly high contribution of genetics in some of the associated 'typically Western' degenerative diseases, which are in fact complex diseases that by definition do not inherit by Mendel's law, illustrating that epigenetic marks can also become erased.

From a pathophysiological point of view, the currently poorly-adapted phenotype in western countries, ensuing from the conflict between the changing lifestyle and our Paleolithic genome, centers on chronic low-grade inflammation and the metabolic syndrome (also named the insulin resistance syndrome); which are risk factors for many of the diseases and conditions typical for affluent countries, such as cardiovascular disease, diabetes mellitus type 2, osteoporosis, certain

types of cancer (notably colon, breast, prostate), fertility problems (polycystic ovary syndrome), pregnancy complications (gestational diabetes, preeclampsia), some psychiatric diseases (major and postpartum depression, schizophrenia, autism) and neurodegenerative diseases (Alzheimer's disease, Parkinson's disease).[20-22] The genetically-determined flexibility to adapt to a changing environment appears to have been exceeded and the genetically most vulnerable have become sick first, but ultimately all individuals will become sick with increasing dose and exposure time.

Environment, nutrients and their interaction with the genome

Adjustment of the DNA base sequence is a slow process that in an individual cannot support adaptation to environmental changes occurring at intermediate or rapid pace. Flexibility for rapid adaptation is provided by genetically-encoded mechanisms that allow adjustment of phenotype by epigenetics and by the interaction of the environment with sensors, such as those of the sensory organs, but also by the many that remain unnoticed.[23-25] The role of nutrients in (epi)genetics and their direct interaction with the genome become increasingly acknowledged.[26] Examples of such nutrients are iodine, selenium, vitamins A and D, and ω3-fatty acids, which are direct or indirect ligands of the thyroid hormone (TR), retinoid X (RXR), retinoic acid (RAR), vitamin D (VDR) and peroxisome proliferator (PPAR) receptors. Homodimerization and heterodimerization of these receptors facilitate gene transcription and thereby keep our phenotype optimally adapted to the reigning conditions of existence. The roles of these nutrients, their respective receptors and the interaction between their receptors are indicative for the importance of their dietary presence and of a certain balance between their dietary intakes to arrive at optimal interaction with the genome. Lessons for this optimal interaction, and hence for the development of randomized controlled trials aiming at the study of diet or lifestyle, rather than single nutrients, might derive from knowledge on human evolution and the conditions of existence to which our ancestors have been exposed. These lessons might provide us with valuable information on what we should genuinely define as a 'healthy diet'.

Evolutionary medicine

The concept that thorough understanding of evolution is important in prevention and treatment of (human) diseases has long been recognized. For example, in the early sixties Theodosius Dobzhansky stated that "nothing in biology makes sense except in the light of evolution"[27], while the Dutch ethologist Nikolaas Tibergen made a distinction between proximate and ultimate (also named evolutionary) causes.[28] Proximate explanations provide a direct mechanism for certain behavior in an individual organism. They explain *how* biomolecules induce certain behavior or for example an allergic reaction. Proximate explanations, however, provide insufficient information to answer the question *why* this behavior or this allergic reaction occurred. Ultimate explanations provide answers explaining *why* things happen from an evolutionary point of view. Many, if not all, diseases can become explained by both proximate and ultimate explanations. The science

searching for the latter explanations has become known as Evolutionary Medicine. Unfortunately, modern medicine deals mostly with proximate explanations,[29,30] while ultimate explanations seem more prudent targets for long-time disease prevention.[29]

The term 'Evolutionary Medicine' (also named Darwinian Medicine) was launched by Randolph M. Nesse and George C. Williams.[31,32] They provided evolutionary answers for the understanding of human diseases. Many diseases do not result from a single biological, anatomical or physiological abnormality, but rather from a complex web of interactions. They often reflect the collateral damage of the survival and reproduction strategies of our genes and the genes of other organisms in our environment. For example, both fever and diarrhea are human *defence-mechanisms* to clear foreign pathogens, while c*onflicts* between a mother and her unborn child, a human and its vegetable food, or a human and an ingested pathogen, can result in pre-eclampsia, celiac disease or auto-immune diseases (streptococcus induced rheumatoid arthritis), respectively. Another example of the influence of evolution in human disease comes from so- called *trade-offs* between human genes and other (hostile) genes in the environment. The most notorious being the survival of the sickle cell gene in malaria endemic regions: the heterozygous carriers (HbAS) have a survival advantage compared to the wild-type (HbAA), despite the death of homozygous subjects (HbSS). Another example is the equilibrium between the not yet full grown, but yet relatively large, brain of a newborn and the small birth canal in its turn is constrained by an upright posture and provides. An often overlooked example is coincidence, which may be illustrated by the blind spot on the back of the retina resulting from the inside-out structure of the eye. Although this inside-out structure is not ideal, a possible turning point has long been past, and we simply have to deal with this imperfection of human evolution. The location of the birth canal on its turn also provides an example of an evolutionary coincidence that urges to deal with an, in retrospect, imperfect evolutionary design. These examples illustrate that evolution builds on the past: it is not possible to start a completely new design from scratch on, which argues against 'intelligent design'. Another important influence of coincidence lies in *exaptation*[33], which is a feature that is not acquired in the context of any function to which it might eventually be put. A well known example comes from feathers which were only used for flying after they had been present for a different purpose, such as thermal insulation, in several species for millions of years. Several human features, such as blue eyes, white skin, our hairlessness and large skull might also have been evolved in another context than they were finally used in. The most important example of an evolutionary explanation for human disease, however, comes from the *mismatch* between our slowly adapting genome and the rapidly changing environment, notably our diet.

Evolutionary medicine argues that the chronic degenerative diseases causing most morbidity and mortality in affluent countries occur because of the current *mismatch* between the rapidly changing conditions of existence and our Palaeolithic genome.[34] These mismatches will persist, notably in the light of our long generation time. The genetic adjustments needed to adapt to the new environment are also unlikely to occur, since the mismatch exerts little selection pressure. That

is, they do not cause death prior to reproductive age, but rather reduce the numbers of years in health at the end of the life cycle.[35] Consequently, Evolutionary Medicine acknowledges a return to the lifestyle prior to the onset of the Agricultural revolution as translated to the culture of the 21th century and as popularized by the expression: "how to become a 21th century hunter-gatherer".[36] Skeptics of Evolutionary Medicine often raise the intuitive criticism that the human ancestor had a very short life expectancy compared to contemporary people.[35] Consequently, they argue, there was no selection pressure on longevity or 'healthy ageing', since there were virtually no old people, while the few individuals reaching old (e.g. postmenopausal) age provided no evolutionary benefit to younger individuals who were still able to reproduce. The counterargument is multileveled.

Arguments and counterarguments in evolutionary health promotion[35]

The first argument provides a quantitative explanation for ageing and states that our genes have simply been selected to make the individual become old, because older individuals generate more offspring, which clearly provides an evolutionary survival advantage. The next arguments, however, provide explanations which emphasize the importance of an increased quality of life in the offspring, rather than their absolute numbers.

It needs emphasis that Evolutionary Medicine predicts no further increase in life expectancy, but rather a decrease in the numbers in deteriorating health at the end of the life cycle. It was estimated that the complete elimination of nine leading risk factors in chronic degenerative diseases would increase life expectancy at birth by only 4 years, since these diseases only affect late-life mortality.[37] Secondly; the increased life expectancy at present originates mostly from the greatly diminished influence of some unfavorable conditions of existence, including (childhood) infections, famine, homicide and tribal wars[34,38] secondary to the high levels of medical sciences and continuing civilization. Thus, to achieve the average life expectancy of 40 years in a present day hunter-gatherer society, for every child that does not survive beyond 1 year of age, another should reach the age of 80. In fact, about 20% of modern hunter-gatherers reach at least the age of 60 years.[39-41] In other words, the popular argument[35] that very few people in these societies live past 50 is unsupported by ethnographic data. The third, often raised, argument is that due to the higher life expectancy in present day humans, it is invalid to compare the mortality figures for cancer and degenerative disease of present day hunter-gatherers (with low life expectancies) with those of Western populations (with a life expectancy of 80 years). However, early biomarkers of degenerative diseases such as obesity, high blood pressure, atherosclerosis and insulin resistance are also less common in younger, age-matched, members of present hunter-gatherer compared to members of affluent societies,[9,42] while measurements indicative for 'good health' such as muscular strength and aerobic power are more favorable in the former[43]. Moreover, even the oldest individuals in hunter-gatherer societies appear virtually free from chronic degenerative diseases.[44-46] A fourth counterargument against the assumption that our human ancestors before the Agricultural Revolution died at young age derives from archaeological records. After the transition from hunting and gathering to farming

about 10.000 years ago, life expectancy dropped from about 40 years (as it is in recently studied hunter-gatherers, but also was among students of the Harvard College Class born in 1880[47] to about 20 years.[48-50] This seemingly evolutionary disadvantage, secondary to a decrease in nutritional quality, is substantiated by a decrease in general health that has become noticeable from a decrease of final height, while skeletal markers of infection and nutritional stress became more common in archaeological finds.[49-52] These setbacks were eliminated by a net increase in population growth, secondary to an increased productivity per land area that resulted in more calories per capita. Life expectancy remained stable throughout the Neolithic until the late 18th century, seldom exceeding 25 years in 'civilized' nations.[35] From this time, improvements in hygiene, food production and manufacturing, energy generation, per capita income, shelter, transportation, clothing and caloric intakes substantiated an increase to and beyond the life expectancy that prevailed before the onset of the Agricultural Revolution. Greater energy availability enhanced e.g. the energy requirements of the immune system and for reproduction, both improving longevity.[35,53] Importantly, it was concluded that medical treatments had little impact on mortality reduction, while public health achievements (sanitation, food and water hygiene, quarantine and immunizations) have critically improved life expectancy. The fifth counterargument is that old people do provide an evolutionary benefit to the younger generations. Male fertility remains largely intact and male provisioning might help in the problem of high female reproductive costs, although the latter is contested.[54,55] The benefits of older females have been put forward in the grandmother hypothesis. This hypothesis, in which the presence of older females within a certain group benefits reproductive success of their offspring, is supported, by studies in human hunter-gatherer[56-62] and primate societies.[56,60,63] Interestingly, these fitness benefits of grandmothering proved insufficient to fully explain the evolution of increased longevity[62], suggesting that other evolutionary benefits, such as grandfathering, might also be involved in the long reproductive and non-reproductive lifespan of *Homo sapiens*. A recent analysis supports such benefits for both older males and females, since the presence of post-reproductive women increased the numbers of newborns by 2.7%, while 18.4% of the infants in a polygamous society in rural Africa was sired by males aged 50 and above.[61] In support of the statement that 'nothing in biology makes sense except in the light of evolution' we therefore conclude that, unless proven otherwise, the presence of a substantial part of older males and postmenopausal females in hunter-gatherer, in contrast to primate societies, should be considered as proof for the evolutionary benefit that these individuals are to their progeny. Finally, we propose that this assumption would only be convincible if these individuals were reasonably fit; thereby supporting the concept of healthy ageing. Hence, healthy ageing seems both supported by ethnographic data and its benefit to hunter-gatherer societies. Other commonly raised arguments against the genome-environment mismatch hypothesis are the potential genetic changes since the Agricultural Revolution, the heterogeneity of ancestral environments and innate human adaptabilty[35]. Counterarguments to these critics have been discussed in great detail elsewhere.[35]

In this review, a multidisciplinary approach is used, including palaeo-environmental

reconstruction, comparative anatomy, biogeochemistry, archaeology, anthropology, (patho) physiology and epidemiology, to assess the characteristics of the ecosystem that supported human evolution. Based on this assessment, an approximation is made of the dietary composition that derives from this ecosystem. Finally, the potential benefit of a return to this 'Palaeolithic diet' is discussed and an update is provided for the evidence for the positive health effects of these diets.

3. Human evolution

Hominins are defined as members of the taxon *Hominini*, which comprises modern *Homo sapiens* and its extinct relatives over the past ~7 million years. The oldest-known hominins **(Figure 1**) are *Sahelanthropus tchadensis* from Chad (~7 million years ago (Mya)[64]) and *Orrorin tugenensis* from Kenya (~6-5.7 Mya[65]). The next oldest are *Ardipithecus kadabba* (Ethiopia, ~5.8 Mya[66]) *and A. ramidus* (Ethiopia, ~4.4 Mya[67]), *Australopithecus anamensis* (Kenya, ~4.1-3.9 Mya[68]), *Au. afarensis* (Ethiopia, Tanzania and may be Kenya, 3.6-3.0 Mya[69,70]), *Au. bahrelghazali* (Chad, ~3.5 Mya[71]), *Kenyanthropus platyops* (Kenya, ~3.5 Mya[72]), *Au. garhi (*Ethiopia, ~2.5 Mya[73]*)* and *Au. africanus* (South Africa, ~2.9 -2.0 Mya[74]). From these earliest hominins evolved the genera *Paranthropus* (3 known subspecies) and *Homo*. The earliest species that have been designated *Homo* are *Homo rudolfensis, Homo habilis* and *Homo erectus sensu lato* –including *H. ergaster* (Eastern Africa, ~2-1.8 Mya): these in turn are the presumed ancestors of Asian *H. erectus*, *H. heidelbergensis* (Africa, Eurasia 0.6-0.3 Mya), *H. neanderthalensis* (Eurasia, 0.4-0.03 Mya) and *H. sapiens* (from ~0.2 Ma onwards)[75-77]. The recently discovered *H.* floresiensis (0.095 - 0.013 Mya[78]) and the previously unknown hominins from Denisova Cave (~0.05-0.03 Mya[79]) show that in the recent past several different hominin lines co-existed with modern humans.

Africa is now generally accepted as the ancestral homeland of *Homo sapiens*.[76,80,81] In several subsequent Out-of Africa waves,[82] hominins of the genus *Homo* colonized Asia, Australia, Europe and finally the Americas (**Figure 2**). Archaic *Homo* species reached as far as the island of Flores in South-East Asia, East-China and Southern-Europe (Spain). *Homo heidelbergensis* remains were found in Africa, Europe and Eastern-Asia, while *Homo neanderthalensis* was restricted to Europe, Western-Asia and the Levant. At last, in the later Out-of-Africa diaspora starting about 100 kya, *Homo sapiens* finally reached Australia and the Americas, while likely replacing earlier hominins in Africa, Europe and Asia that had left during the earlier Out-of-Africa waves. However, there remains some debate[81,83-85] whether or not the gene pool of archaic hominins contributed to that of modern humans. In the replacement theory, archaic hominins make no contribution to the gene pool of modern humans, whereas in the hybridization theories (either through assimilation or gene flow), newly arriving hominins from the later Out-of-Africa wave mixed with archaic predecessors. Current evidence from DNA analyses supports the concept that the gene pool of archaic hominins, notably Neanderthals,[86] but also Denisovans[79] contributed to the gene pool of *Homo sapiens* (**Figure 3**).

The African cradle of human kind is supported by micro-satellite studies[87] that reveal that within populations the genetic variation decreases in the following order: sub-Saharan Africa>Eurasia>East

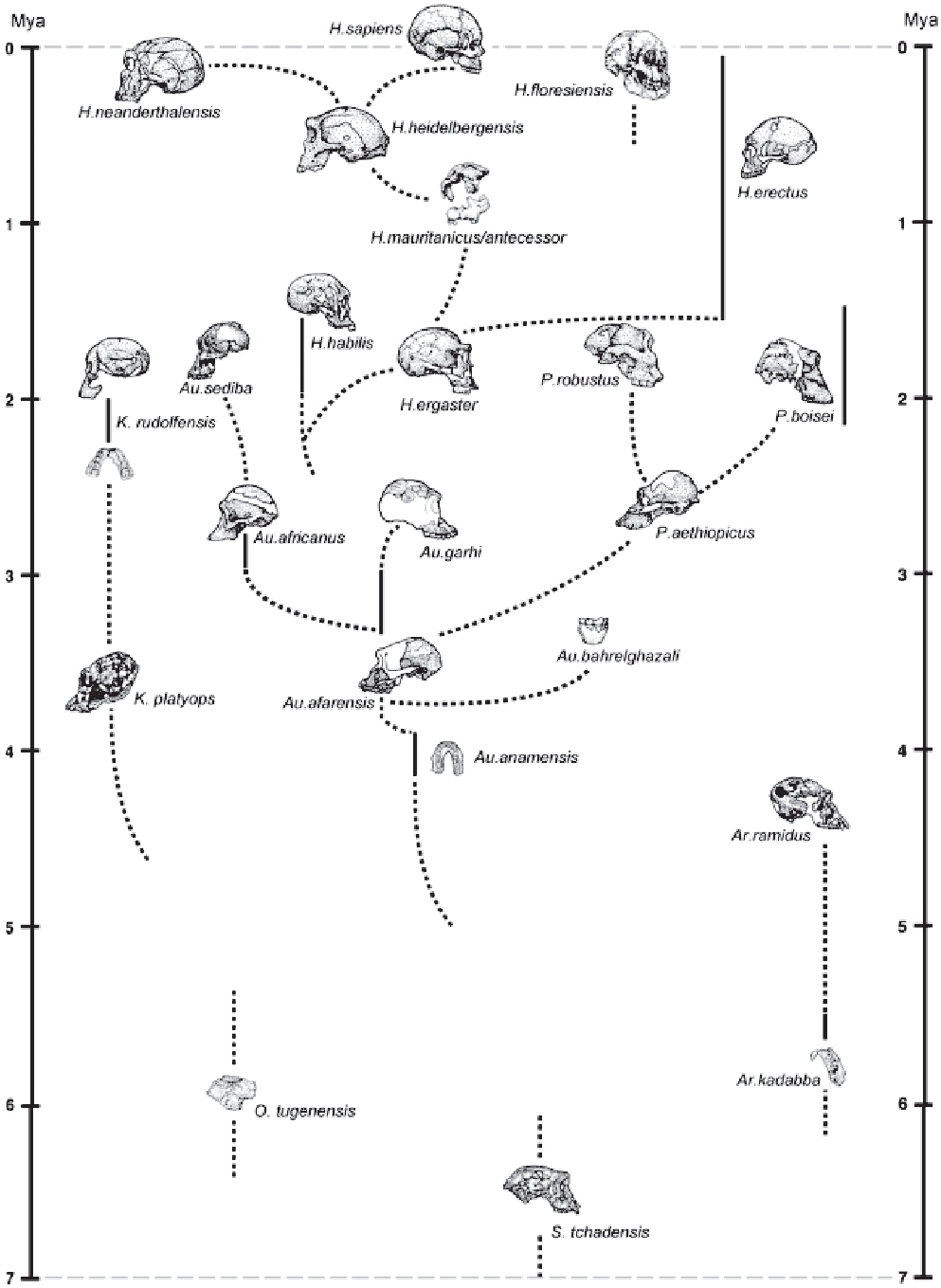

Figure 1. Scheme of the possible phylogenetic relationships within the family Hominidae. Note that at many time points of evolution, several different hominin species coexisted. © Ian Tattersall, with permission. [63]

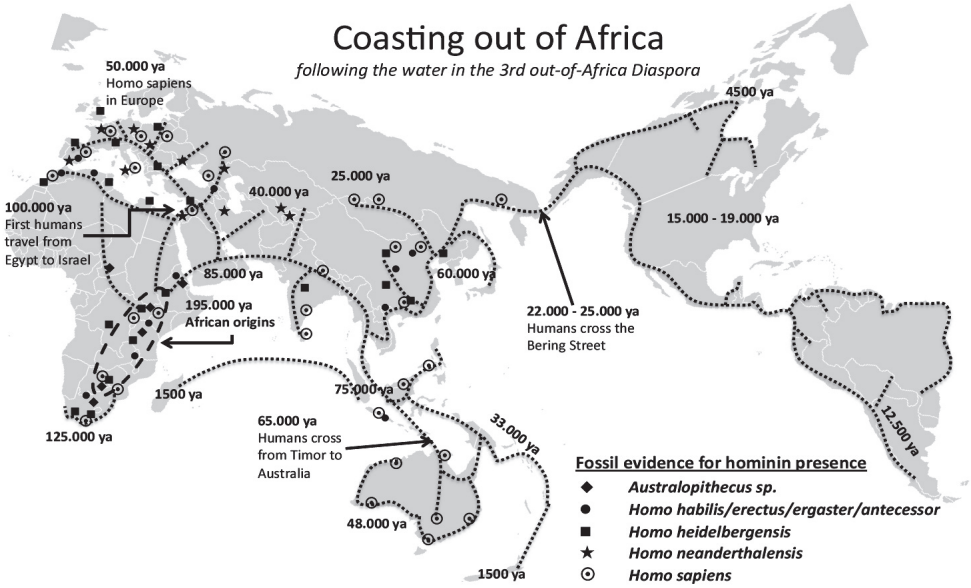

Figure 2. Coasting out of Africa. The assumed dispersal routes of archaic and anatomically modern humans out of Africa and the supportive fossil evidence for hominin presence. Source: National Geographic Society 1988, 1997; adapted from www.handprint.com/LS/ANC/disp.html and Oppenheimer.[68.]

Asia>Oceania>America, with the hunter-gatherer Hadzabe of Tanzania separated from the Ju|'hoansi (previously called !Kung) from Botswana by a genetic distance greater than between any other pair of populations,[88] which indicates the chronology of continent inhabitation and points to South or East Africa as the cradle of humankind.[88,89] Human evolution was characterized by several large-scale decimations, and it has been estimated that the current world population derives from only 1,000 surviving individuals at a certain time point.[90] Such bottlenecks[91], characterized by strong population decrease, or where groups of hominids were separated due to global climate changes, volcanic winters or geographic boundaries as mountain ridges or seas, caused *gene flow* and *genetic drift*. As a result different phenotypic races emerged in different geographic regions.[87,91] However, differences among these populations contribute only 3-5% to genetic diversity, while within-population differences among individuals account for 93-95% of genetic variation.[92] In other words, genetically we belong to one species that originally evolved in Africa and that for the great majority genetically still resides in the Palaeolithic era. Most of the current inter-individual genetic differences were already existent when *Homo sapiens* emerged, some 200,000 years ago.[77] Bipedalism, hairlessness, speech and the ability to store fat differentiates humans from the closest relatives, the primates, but it is the uniquely large brain, which allowed for symbolic consciousness and pose 'what if' questions, that finally made humanity.[75]

3.1 Changing habitat and increasing brain size

It is assumed that during the early stages of human evolution early hominins introduced more animal food into their diets, at the expense of plant foods.[93,94] Subsequent hominins further increased the amount of animal food and consequently the energy density and (micro)nutrient content of their diet, i.e. the dietary quality (DQ). While increasing their dietary intake from animal food, early hominins grew taller and increased their brain mass relative to body mass (encephalization quotient, EQ). Brain mass in primates relates to the number of neurons[95] and global cognition,[96] while the human cortex also has more cycles of cell division compared to other primates[97]. During hominin evolution the first significant increase in EQ occurred around 2 Mya (**Table 1**). From ~2 million to 200-thousand years ago (kya) the human ancestors tripled their brain size from *Australopithecus* species with an EQ of 1.23-1.92 to an EQ of 1.41-4.26 for the genus *Homo*.[98,99] The increase in brain size and the number of neurons differentiates *Homo* from their closest primate relatives. However, a large brain requires an adaptation or an exaptation to accommodate it, and notably sufficient intake of so-called 'brain-selective nutrients'[99,100] to build and conserve it.

3.2 Buiding a big brain

Compared to other primates, humans have extraordinarily large brains.[101,102] To understand the expansion of the human brain during evolution, it is important to comprehend its composition and its biochemistry. Brain tissue has a unique profile of long-chain polyunsaturated fatty acids (LCP)[98]. Comparison of the brain ethanolamine phosphoglycerols of 42 studied animal species shows an

Table 1. The development of brain weight relative to body dimensions[a]

Species	Brain weight (g)	Brain-to-body ratio (%)	Relative EQ[b]
Gorilla gorilla	500	0.3	25
Pongo pygmaeus	400	0.5	32
Pan troglodytes	400	0.9	42
Australopithecus afarensis	455	1.7	41
Australopithecus africanus	450	1.0	44
Paranthropus aethiopicus	405	1.1	44
Paranthropus boisei	510	0.9	46
Paranthropus robustus	520	1.1	50
Homo rudolfensis	750	1.7	59
Homo habilis	600	1.7	57
Homo ergaster	855	-	
Homo erectus	863	1.6	63
Homo heidelbergensis	1200	1.8	74
Homo neanderthalensis	1450	1.9	75
Homo sapiens (Cro-Magnon)	1490	2.4	102
Modern Homo sapiens	1360	2.3	100

a, adapted from Cunnane [99]
b, relative to modern Homo sapiens

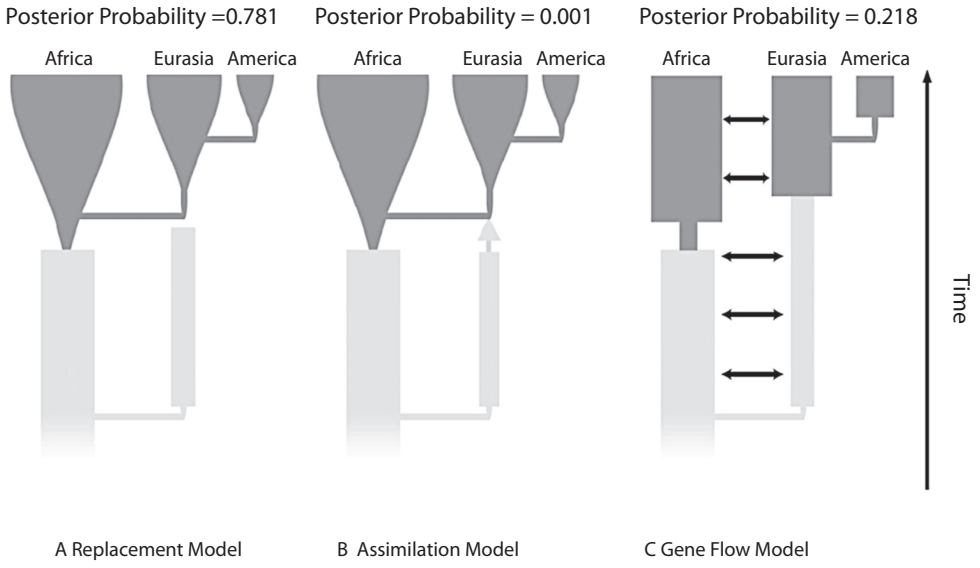

Figure 3. The three models of human evolution and their posterior probabilities. Dark grey represents anatomically modern humans, lighter gray the archaic populations. The width of the gray areas indicates the population size over time. The arrow in B indicates the possibility of a genetic contribution of archaic humans to modern populations via admixture. The double-headed arrows in C indicate gene-flow between two populations. Adapted from A. Templeton, with permission.[83-85]

almost identical LCP pattern, independent of the grade of encephalization, containing approximately equal proportions of arachidonic (AA) and docosahexaenoic (DHA) acids. Consequently, for normal neuronal function, mammalian brain tissue appears to have an invariant structural requirement for both AA and DHA. This shows that both these fatty acids are important building blocks for building a big brain and for encephalization. The weight of a newborn human brain is about 340 g[103] and it contains about 9 g lipid[104]; the brain of a 10 months old infant is 850 g and contains 52 g lipid. At 3 years, the brain is 1,100 g and contains 130 g lipid. Thus, the major part of the human brain spurt occurs postnatally,[105] implying that especially the newborn infant has high demands for AA and DHA.

Toothed whales (brain weight 9,000 g) and African elephants (4,200 g) have brains much larger than humans, but they have lower cognitive abilities and a lower EQ.[106] These observations substantiated an EQ-centered approach to explain variation in cognition between species. Recent analyses, however, showed remarkable differences between primate and non-primate brains, assuring that primate brain contains many more neurons than a non-primate brain of similar size[95,107,108] and that the absolute number of neurons, rather than body relative to brain ratio (EQ) best predicts cognitive ability[96]; although it still needs to be determined whether humans have the largest number of brain neurons among all mammals. From this new neuron-centered view, there seems to be nothing special about the human compared to the primate brain, except for

its size,[95] which basically determines both the number of neurons and non-neurons.[109,110] Detailed comparisons of human and primate brains revealed other differences, such as different levels of gene expression,[111-113] secondary to chromosomal rearrangements,[114] differences in the relative extent of the neocortical areas[95,115], the distribution of cell types[116] and the decrease of brain structure volumes with increasing age in humans in contrast to chimpanzees.[117,118] The best predictor of cognitive ability in humans compared to non-primates, however, still needs to be established, but rather than EQ or brain size, the absolute number of neurons seems a prudent candidate,[96,107] since there is no clear relation between neuron number and the absolute brain size among the different animal species.[95,107,108]

In contrast to intuitive belief, growing a large brain and a large skull to accommodate it is less difficult to achieve than it seems at first glance. It was recently shown that different levels of expression of a single gene might have resulted in the markedly different beak shapes and lengths of Darwin's finches. Experimental overexpression of the calmodulin gene in chicken embryos resulted in a significant increase in the length of their beaks.[119,120] These experiments suggest that small and seemingly insignificant changes can have profound implications for the evolution of anatomical size and shape and thereby provide great potential for explaining the origins of phenotypic variation,[121] including increases in brain and skull size. Analogously, many mutations in humans are associated with either microcephaly[122] or macrocephaly,[123] while the growth of the skull in hydrocephaly shows that the increased skull-size is secondary to the increase of its contents, suggesting that brain rather than skull size is the limiting factor here. The evolution of certain genetic variants associated with brain size has accelerated significantly since the divergence from the chimpanzee some 5-6 million years ago. A recent variation that occurred 37,000 years ago, has spread more rapidly through the human population than could be explained by genetic drift,[124-127] suggesting that it conferred evolutionary advantage.

The anatomical and metabolic changes encoded in the genome (see 'Comparative anatomy') might have provided hominins with the anatomical and energetic opportunity to, over a period of several million years, steadily increase their brain size, but these mutations *per se* did not fulfill the nutrient requirements for brain expansion.[100,128-130] The underlying small number of mutations should rather have been accompanied, and most probably have been preceded, by increased availability of 'brain specific nutrients' such as LCP for their ultimate conservation through the process of mutation/selection, which basically underlines both Darwin's concept of the crucial importance of 'the conditions of existence' and the secondary role of mutation. An example may come from the current knowledge on the sources of AA and DHA. In humans, both AA and DHA can be synthesized from their precursor essential fatty acids alpha-linolenic (ALA) and linoleic (LA) acids (**Figure 4**), respectively. ALA and LA are present in various natural food resources. ALA is predominantly found in plant foods, while LA is mainly found in vegetable oils such as sunflower oil. Both AA and DHA may derive from their synthesis from abundantly consumed precursor fatty acids ALA and LA, but in humans and especially neonates, these synthetic activities are insufficient to cope with metabolic

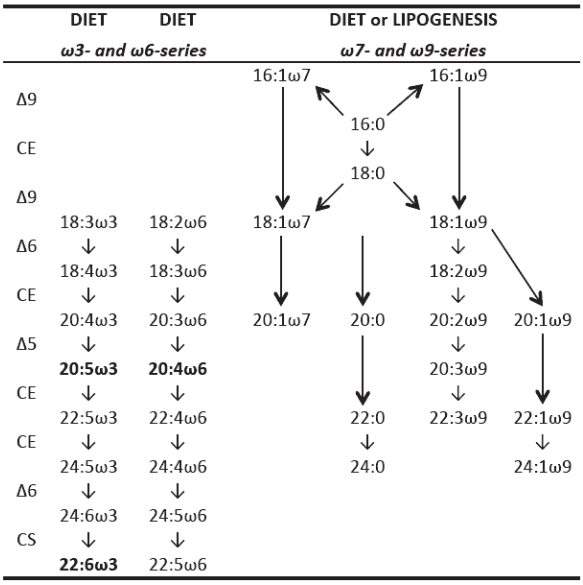

Figure 4. Metabolism of dietary fatty acids and endogenously synthesized fatty acids. Abbreviations: Δx: Δx-desaturase; CE: chain elongation; CS: chain shortening through peroxisomal β-oxidation. 18:3ω3, α-linolenic acid (ALA); 18:2ω6, linoleic acid (LA); 18:1ω9, oleic acid; 20:5ω3, eicosapentaenoic acid (EPA); 20:4ω6, arachidonic acid (AA); 20:3ω9, Mead acid; 22:6ω3, docosahexaenoic acid (DHA).

demands.[131] Consequently both these long-chain polyunsaturated fatty acids (LCP), but especially those of the ω3 series, need to be present in sufficient quantities in our diet. It is still under debate what dietary resource(s) provided the LCP that enabled us to grow a large brain.[100,132-136]

4. The probability of hunting on the savanna

It has been a longstanding paradigm in palaeoanthopology that early human evolution occurred in a dry and open savanna environment.[137-139] Recent studies from the Afar Basin,[67,140] although recently contested,[141-143] indicated that the habitat of *Ardipithecus ramidus* at ~4.4 Ma was characterized not by savanna but by woodland to grassy woodland conditions. Human characteristics, such as the poor water drinking capacity, excessive urination and transpiration and poor water retention support the argument that we would be poorly-adapted savannah dwellers.[139]

A second long reigning paradigm was "Man the Hunter", which was the standard version of human origins advocated for many years. Washburn and Lancaster[93] referred at most to our most recent antecessors, *Homo sapiens* and possibly *H. neanderthalensis*, when they claimed that our intellect, interests, emotions and basic social life are evolutionary products of the hunting adaptation. The strongest argument against this hunting paradigm comes from combined studies of past and present day hunter-gatherer societies indicating that the role of hunting is exaggerated, notably (around the campfire) in hunter-gatherer societies, since the majority of the dietary protein is in reality obtained by women gathering nuts, tubers and small animals.[144-146] Cordain et al.[147] showed

that only 25-35 energy% (en%) of subsistence in worldwide hunter-gatherer communities derived from hunting, while the remainder derived from both plant and fished food. Thus while meat from large game may have been the most *valued* food, it is highly unlikely that it was the most *valuable* (nutritionally important) food resource from a dietary perspective.[41,148] At present, the niche of early hominins and thus the environment of human evolution, and most importantly for this review, the nutritional composition of the early human diet, is still heavily debated.[149]

5. Reconstruction of our ancient diet

In next paragraphs we will discuss various views on (changes in) the hominin ecological niche that over time shaped the human genome to what it currently is.

5.1 Palaeoenvironments

Sahelanthropus, Orrorin and Ardipithecus

In the late Miocene (up to 5.3 Mya), the African continent became more arid, which resulted in fragmenting of the (sub)tropical forests and the appearance of more open environments.[150] The widespread dispersal of some of the earliest hominins such as *Sahelanthropus,*[64,151] and *Australopithecus bahrelghazali* from Chad, might be explained by the presence of the relatively low-lying humid East-West corridor constituted by the remnants of the Cretaceous Central African and Sudan Rifts between western and eastern Africa.[152,153] The reconstructed environment of *Sahelanthropus* (~7 Mya) suggests a mosaic from gallery forest at the edge of a deep, well-oxygenized lake, swampy and vegetated areas, and extensive grasslands.[154] Since there is no indication of carnivore modification or fluvial transport of its bones, *Sahelanthropus chadensis* likely lived in this area.[155] The palaeo-environment of *Orrorin* (~6 Mya) was probably characterized by open woodland; with dense stands of trees in the vicinity and possibly fringing the lake margin and/or streams that drained into the lake.[156] *Ardipithecus kadabba* (5.6 Mya) remains are associated with wet and closed, grassy woodland and forest habitats around lake or river margins.[157] *Ardipithicus ramidus* (4.4 Mya) lived in or near a groundwater-supported grassy woodland to forest.[158] Additionally, the abundance of fossilized shallow-water aquatic species such as catfish, barbus, cichlidae and crocodiles additionally suggest an episodically present flood plain environment.[158]

Early Australopithecus species

Australopithecus anamensis appeared at ~4.2 Mya and its environment was characterized by a mix of wetlands and terrestrial environments, such as lacustrine and fluvial floodplains, woodland and gallery forest.[155,159-162] The later *Australopithecus afarensis* survived in a variety of habitats,[163] but apparently thrived better in the more wooded and humid conditions in the Afar Basin than in the relatively dry Laetoli area.[164] Stewart[155] pointed out that in Africa the only environmental constant in hominin sites throughout the period from 3.4-2.9 Mya was a wetlands habitat, characterized by aquatic herbaceous vegetations around lakes and rivers, with large populations of wetland fauna

such as reduncines and hippos. Hence, these wetlands could have been refugee for early hominins throughout an extensive period of human evolution.

Paranthropus, late *Australopithecus and Homo species*

About 2.9-2.5 Mya tectonic and global climatic changes made Africa cooler and drier.[165-168] The great wet forests of middle Africa retreated and made place for more savannah grasslands. It is around this time, from ~2.6 Mya onwards, that the first traces of the new hominin genus *Homo* appeared in the archaeological record.[139] It has been suggested that alternating wet and dry periods after 2.7 Mya could have isolated hominin populations around sources of potable water, while forcing them to the extremes of their conditions of existence[155] and thus facilitating specialization,[169] either by adaptation or exaptation. Compared to *Australopithecus*, *Paranthropus* existed in slightly more open habitats, including wetlands and grasslands, but also in woodland and bushland areas. The habitats of *Homo* species seems similar to those utilized by *Paranthropus* species,[160] but *Homo* remains at Olduvai Gorge and Koobi Fora are associated with well-vegetated swamps, lakes and river margins, and (semi-) aquatic fauna.[170,171] Only the later *Homo* species are also found in assemblages that indicate extremely arid and open landscapes such as savannah.[160] Also, it has been suggested that hominins and other (aquatic) species dispersed throughout Africa along water systems, while even the last Out-of-Africa migration might have occurred via the "green Sahara" that existed during the last interglacial (125 kya).[155,172]

In conclusion, the palaeo-environmental evidence suggests that early hominins lived in the proximity of water. However, it is frequently argued that bones are preferentially preserved in lake, river or fluvial sediments making their recovery in any other than an aquatic setting unlikely.[173] Alternatively, hominin remains may have been relocated to the water by carnivores,[161] including crocodiles. Nevertheless, the combined evidence strongly suggests that early hominins frequented the land-water ecosystem and thus lived there. Joordens et al.[153] proposed, based on comparison with other terrestrial omnivores, that the default assumption should be that hominins living in freshwater or marine coastal ecosystems with catchable aquatic resources, could have consumed these aquatic resources.[153,174]

5.2 Comparative anatomy

The diet of our closest relatives

Field studies on our closest relatives, the extant apes, show that their preferred food items are primarily fruits and/or leaves and stems from terrestrial forests. Lowland Gorillas for example derive 57% of their metabolizable energy (en%) from short-chain fatty acids derived from colonic fermentation of fiber, 2.5 en% from fat, 24 en% from protein and 16 en% from carbohydrate.[175] "Fallback foods" are consumed when preferred foods are unavailable[176] and are generally composed of herbaceous plants and high fiber fruits from aquatic and terrestrial environments.[155] Like our closest relatives[155,177-180] hominins might have used foods from the aquatic environment as fallback

foods, while, although speculative, this niche might eventually have proven favorable with regard to subsequent encephalization.

Teeth morphology and dental microwear

Comparative anatomy (**Figure 5**) of the hominins might confer some information about these fallback and preferred foods of our ancestors. Dental studies of *Sahelanthropus* (Figure 1) describe that it had thick enamel,[64] similar to orang-utans, suggesting that it could eat hard and tough foods,[181] such as available from the lakeshore vegetation.[155] *Ardipithecus ramidus*, however, had thin molar enamel and smaller teeth compared to later hominins. This dental morphology is consistent with a partially terrestrial, omnivorous/frugivorous niche.[182] Studies on cranio-dental changes such as tooth size, tooth shape, enamel structure and jaw biomechanics indicate that *Australopithecus* and *Paranthropus* had prominent jaws, relatively flat molar teeth, small incisors and thick enamel, suitable for breaking and crushing small hard, brittle foods such as fruits, nuts and underground storage organs,[149] but unsuitable for breaking down tough plant foods or tearing meat. Together, this would allow early hominins to eat both hard and soft, abrasive and non-abrasive foods, which suits well for life in a variety of habitats.[183]

In addition to studies on teeth morphology, micro-wear studies are essential. Dental microwear studies analyse tooth-wear, showing evidence for where teeth were actually used for and thus what an animal in reality ate.[184] While adaptive morphology will give important clues about what a species was capable of eating, microwear studies reflect what an animal ate during some point at its lifetime. In these studies, "complexity" is used as an indicator for hard and brittle items, while "anisotrophy" is an indicator for tough foods.[185]

Most primates show either low complexity combined with high anisotrophy indicative for consumption of tough foods such as leaves, stems and meat; or high complexity with low anisotrophy associated with hard-brittle foods, such as nuts and seeds.[149,186] *Ardipithecus ramidus*' preference

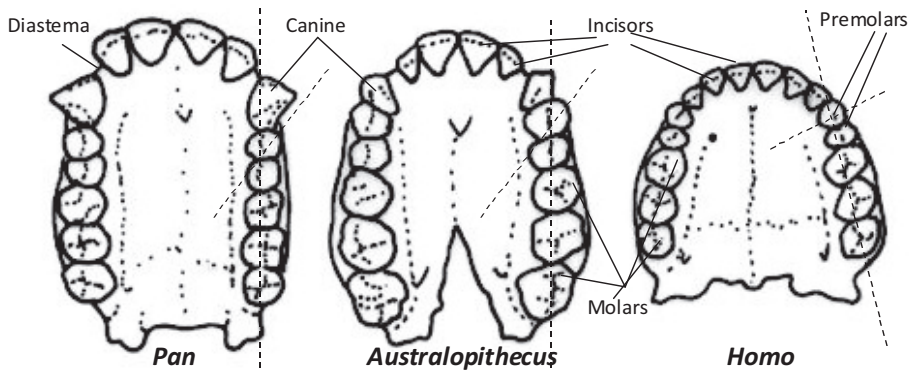

Figure 5. Lower jaw of a chimpanzee (Pan troglodytes), Australopithecus africanus and Homo sapiens. Note the somewhat human-like shape of the teeth, but ape like axis in the jaw of Australopithecus. © Australian Museum.

for an omnivorous/frugivorous diet was confirmed by microwear studies[182]; suggesting a diet of fleshy fruits and soft, young leaves.[149] Conversely, microwear textures of *Australopithicus afarensis* and *anamensis* show striations rather than pits (low complexity and low anisotrophy), i.e. patterns similar to those of grass-eating and folivorous monkeys instead of the predicted diets predominated by hard and brittle foods.[186,187] *Australopithecus africanus* showed microwear patterns that were more anisotropic, suggestive for consumption of tough leaves, grasses and stems.[188] *Paranthropus robustus*, also know as the "Nutcracker Man," has enormous, flat, thickly enamelled teeth, that are combined with a robust cranium, mandible and powerful chewing muscles, suggestive for breaking hard and brittle foods.[185] After microwear analysis of its teeth, however, Ungar et al.[149,185] showed that *P. robustus* had low complexity and anisotrophy, thus Paranthropus might only have consumed mechanically challenging items as fallback foods when preferred foods were unavailable. Similarly, microwear studies support the notion that the diet of *P. boisei* contained large quantities of low-quality vegetation, rather than hard objects.[189]

Generally, micro-wear studies confirm earlier dental topography studies,[190] which revealed the incorporation of more fracture-resistant foods, i.e. tougher foods as leaves, woody plants, underground storage organs and animal tissues, in the diet of *Australopithecus africanus* compared with *Australopithecus afarensis* and for *P. robustus* compared with *Australopithecus africanus*.[186] Dental topographic analysis suggested that successive *Homo* species emphasized more on tougher and elastic foods, perhaps including meat.[190] The latter suggestion is in line with the optimal foraging theory, which states that humans prefer foods with high energy density over those with low energy density.[191,192]

Microwear studies confirmed that early *Homo*, such as *H. erectus* and *H. habilis* did not prefer fracture resistant foods, although some *H. erectus* specimens showed more small pits than *H. habilis* members, suggesting that none of the early *Homo* specialized on very hard-brittle or tough foods, but rather could consume a varied diet.[193] This does not imply that early *Homo* had very broad diets, but rather that early *Homo* was adapted to subsist in a range of different environments, providing evolutionary advantage in the climatic fluctuations and the mosaic of habitats in Africa during the late Pliocene.[194] A study on dental microwear of 300,000 year old *H. heidelbergensis* teeth from Sima de los Huesos in Spain, showed striation patterns that indicated a highly abrasive diet, with substantial dependence on poorly processed plant foods such as roots, stems and seeds.[195] Lalueza et al.[196] compared teeth of very recent hunter-gatherers (Inuit, Fueguians, Bushmen, Aborigines, Andamanese, Indians, Veddahs, Tasmanians, Laps and Hindus) with Middle and Upper Pleistocene fossils. Their results indicate that some Neandertals resemble carnivorous groups, while archaic *H. sapiens* show a more abrasive diet, partly dependent on vegetable materials.

Overall, remarkably few studies have related microwear patterns to hominin diet for the period between 1.5 million years and 50,000 years ago *(Ungar, pers. communication)*. Studies in more recent hominins, such as from an Upper Palaeolithic site in the Levant (22,500-23,500 BP) showed a high frequency of long narrow scratches and few small pits, suggesting a tough abrasive diet of

aquatic foods rather than a diet with hard foods that needed compressive force.[197,198] A study of subsequent local hunter-gatherers (12,500-10,250 BP) and farmers (10,250-7,500 BP) living in the Levant showed larger dental pits and wider scratches among the farmers compared to the hunter-gatherers, suggesting that the implementation of agriculture led to a more fracture resistant diet.[198]

Gut morphology, energy expenditure and muscularity

Gut morphology studies[199,200] support the introduction of animal foods at the expense of vegetable foods, in the diet of early *Homo*. The dominance of the colon (>45%) in apes indicates adaptation to a diet rich in bulky plant material, such as plant fiber and woody seeds. In contrast, the proportions of the human gut, dominated by the small intestine (>56%), suggests adaptation to a diet that is highly digestible, indicating a closer structural analogy with carnivores than to folivorous or frugivorous mammals. Importantly, the shorter gut in *Homo*, as compared to primates, might have had some other advantage. During the evolution from *Ardipithecus* and *Australopithecines* to early *Homo*, the improvement of DQ coincided with an increase in height, the size of our brain and its metabolic activity. However, an increase in body size coincides with increased daily energy demands, notably during gestation[201] and lactation.[202] It was e.g. calculated that daily energy expenditure (DEE) for a *Homo erectus* female is about 66% higher compared to an *Australopithicine* female, while being almost 100% higher in a lactating *Homo erectus* female compared to a non-lactating, non-pregnant *Australopithicine*.[203] These high energy demands might have been met by increased female fat reserves,[204,205] such as demonstrated by the presence of female steatopygia in some traditional human populations, such as the Khoisan of southern Africa **(Figure 6)**.

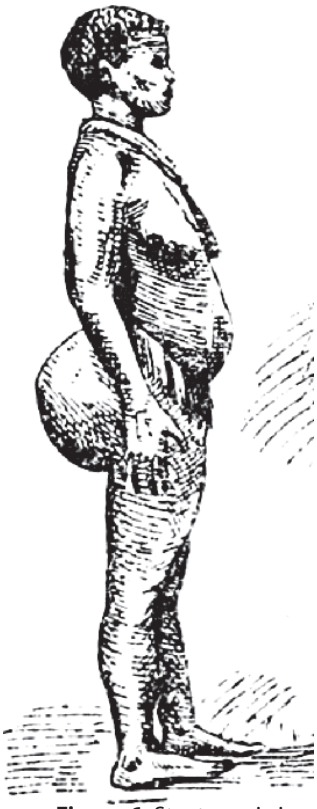

Figure 6. Steatopygia in Hottentot woman

Apart from the extra energy need for reproduction and increasing height, the human brain of a modern adult uses 20-25 en% of the total resting metabolic rate (RMR), while this value is 8-9 en% for a primate.[206] It has been postulated that the extra energy needs did not derive from a general increase in RMR, but partly from a concomitant reduction of the gastrointestinal tract[207] and a reduction in muscularity.[206] These features are combined in the 'expensive tissue hypothesis' of Aiello and Wheeler[207] that points at the observation that the mass of the human gastrointestinal tract is only 60% of that expected for a similar-sized primate and that humans are relatively under-muscled compared to other primates.[206] Interestingly, the negative relation between gut and brain size across anthropoid primates[208] was confirmed in a study with highly encephalized

fish,[209] whereas it became falsified in mammals.[101] Unfortunately, however, the latter study did not include marine mammals,[101] while it is questionable whether with respect to brain development humans adhere to general mammalian rules.[102] Other adaptations might have saved energy expenditure as well. The short human inter-birth interval,[203] compared to inter-birth intervals of 4-8 years in gorilla, chimpanzee and orangutang[210] reduces the most expensive part of reproduction, i.e. lactation,[211,212] while the shift from quadrupedal to bipedal locomotion also reduced DEE.[213] Together, all of these adaptations allow for an increased DEE, including the reallocation of energy to the metabolically active brain, but were only possible after *Homo* included more energy dense foods into its diet. Brain mass in primates is positively related to DQ and inversely to body weight.[206] Generally, a shift towards more energy dense foods includes a shift from primarily carbohydrate-rich vegetables to fat- and protein-rich animal foods. However, it has also been suggested that a shift from the complex carbohydrates in leafy vegetables towards underground storage organs, such as tubers, might have provided easy to digest carbohydrates[199,214] to support a larger hominin body mass.

A phenotypic specialization on non-preferred resources, without compromising the ability to use preferred resources, is also known as Liem's Paradox.[215] This paradox is important to keep in mind during attempts to reconstruct the *preferred* diet from the available evidence. Interestingly, Stewart[155] recently noted that in the case of *Paranthropus*' phenotype with regard to its fallback food, there is just as much evidence to talk about a "Nutcracker Man", as there is to talk about a "Shellcracker Man". Thus, a phenotypic characterization needs support from other studies to confirm adaptation to preferred rather than fallback foods. In other words, it should be noted that not all physical characteristics might be taken as unambiguous proof for the preferred diet of early hominins (Liem's Paradox). The increasing absolute body size and brain size and the reduction in gut size, however, do indicate a shift from low to high DQ foods for more recent hominins.

5.3 Biogeochemistry

Evidence from the strontium/calcium ratio

Based upon the principle 'you are what you eat'[216] several techniques were developed to study early hominin diets[217]. Trace element studies first started with strontium to calcium ratios (Sr/Ca), which decrease as an animal moves up the food chain, secondary to the biological discrimination against strontium.[218] A first study[219] suggested that *Paranthropus robustus* (Figure 1) had lower Sr/Ca ratios compared to contemporaneous *Papio* (baboon) and *Procavia* (hyrax), suggesting that *Paranthropus* was not an exclusive herbivore. However, a subsequent study showed that two *Homo* specimens had a higher Sr/Ca ratio than *Paranthropus.*[220] Since *Homo* had been assumed to consume more animal foods than *Paranthropus* and thus have a lower Sr/Ca ratio, these higher Sr/Ca ratios needed explanation. For example, consumption of specific foods with a high Sr/Ca ratio might have increased the ratio in *Homo* compared to *Paranthropus.* Foods in the area of research with elevated Sr/Ca ratios were mainly geophytes. These are notably perennial plants with underground food

storage organs, such as roots, bulbs, tubers, corms, or rhizomes. Consequently, this discrepancy has been attributed to the consumption of underground storage organs by early *Homo*.[214,220] The use of these underground storage organs may have become necessary from the start of a first period of aridity around 2.8 Mya, when forest was replaced by drier woodlands, forcing hominins to search for the available resources around water margins.[221]

Although promising at first, the use of Sr/Ca ratios was found to suffer from several limitations. For example, a problem is that hominin Sr/Ca ratios in fossilized bones alter with time;[222] a process that is known as diagenesis. This problem can be circumvented by the use of tooth enamel, which is less susceptible to diagenesis than bone.[222] Subsequent studies showed that *Australopithecus africanus* had a higher Sr/Ca ratio in its enamel compared to *Paranthropus* and contemporaneous browsers, grazers, baboons and carnivores.[223-225] The interpretation of these findings, however, remains a subject of debate. For example, a group of brown seaweeds and some aquatic plants discriminate against calcium, resulting in an increase in the Sr/Ca ratio, and likewise in the Sr/Ca ratio of the fish feeding on them.[225,226] Similarly, leaves from trees have lower Sr/Ca ratios compared to grasses, which becomes subsequently reflected in the Sr/Ca ratios of browsers and grazers, respectively.[227] Thus, it might be argued that the relatively high Sr/Ca ratios in *Australopithicines* and *Homo* reflect their consumption of aquatic resources or of animals such as insects or other small animals feeding on grasses. For now, the exploration of especially Sr/Ca ratios as a dietary proxy method has largely been stalled.[228]

Evidence from the barium/calcium ratio

Another trace element ratio that might provide information on the composition of the early hominin diet is the barium to calcium ratio (Ba/Ca), particularly when used in a multiple element analysis with Sr/Ca and Sr/Ba ratios.[223,229] Combined Ba/Ca and Sr/Ba ratios clearly differentiate grazers from browsers and carnivores in both modern and fossil mammals[229]. Hominins have a lower Ba/Ca and higher Sr/Ba ratio compared to grazers and browsers. *Paranthropus* shows considerable similarity for both ratios with both carnivores and *Papionins* (baboons), while *Australopithecus* shows an even higher Sr/Ba ratio. The unusual combination of a high Sr/Ca and low Ba/Ca ratio in hominins (and baboons) was further observed for some animals as warthogs and mole rats that make extensive use of underground resources.[230] Finally, the high Sr/Ba ratio, as observed in *Australopithecus*, might derive from the consumption of grass seeds, which have Sr/Ba ratios 3-4 times higher than grass straw, while consumption of these grasses is also consistent with stable isotope evidence (see below) showing that *Australopithecus* derived a substantial part of its diet from C4 resources. Although not included in any study so far, Sr/Ba ratios in aquatic foods might add to the understanding of the unusual combination of low Ba/Ca and high Sr/Ba ratios.

Evidence from the $^{13}C/^{12}C$ ratio

Fractionation studies of carbon isotopes differentiate between different routes of photosynthesis.

While most tropical African woody plants from forests (like fruits, leaves, trees, roots, bushes, shrubs and forbs) use the C_3-photosynthetic pathway, some South-[231] and East-African[232] grasses and sedges, use the C_4-photosynthetic pathway. C_4-plants such as sedges (e.g. Cyperus papyrus) typically occur in a mosaic of extensive seasonal and perennial shallow-freshwater wetlands that can also be found in savannah and 'bushvelds' receiving summer rainfall.[233] The real impact of C_4-plants occurred with their spread into Eastern and Southern Africa during the Pliocene.[234] Tissues of plants that utilize the C_4-pathway have a relatively high content of the stable carbon isotope ^{13}C (about 1.1% of carbon), since C_3-plants discriminate more strongly against $^{13}CO_2$ during photosynthesis. As a result C_3- and C_4-plants have quite different $^{13}C/^{12}C$ ratios in their tissues, as have the herbivorous animals that feed on these plants[227,231] and the carnivores that prey on these herbivores.[235,236] Differences are expressed as $\delta^{13}C$ values ($\delta^{13}C$ (in ‰) = $[(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}]*1000/(^{13}C/^{12}C)_{standard}$) in parts per thousand (‰) relative to the $^{13}C/^{12}C$ ratio in the reference standard (named Pee Dee Belemnite, PDB), i.e. the carbonate obtained from the fossil of a marine Cretaceous cephalopod (Belemnitella Americana) which is highly enriched in ^{13}C ($^{13}C/^{12}C$ ratio=0.0112372). Consequently, most animals have negative $\delta^{13}C$ values (**Figure 7**). The $\delta^{13}C$ ranges from -35 to -21‰ (mean -26‰) in C_3-plants and from -14 to -10‰ (mean -12‰) in C_4-plants.[224] Studies that attempted to reconstruct mammalian food webs, indicated that carbon is slightly enriched (1-2‰) with each trophic step.[193,206] To facilitate comparison of current and historical animals, $\delta^{13}C$ analyses are predominantly performed in hard tissues, such as bone collagen and enamel (notably apatite), since these constitute the majority of the fossil record. It was shown that enamel mineral is enriched by about 13‰ compared with dietary $\delta^{13}C$[237], while collagen is enriched by about 5‰ compared with dietary $\delta^{13}C$[224]. Collagen from terrestrial mammal C_3-herbivores shows a value of -21‰ (range -22 to -14‰), while in collagen of C_4-herbivores this value is -7‰ (-12 to -6‰). Their respective carnivores show collagen values of -19‰ (-21 to -14‰) and -5‰ (-8 to -2‰), respectively[224]. Marine phytoplankton, which uses the C_3-pathway, shows an average value of -22‰ (238). Collagen of marine fish shows a range from -15‰ to -10‰, while the values in collagen of reef fish range from -8‰ to -4‰[239]. Marine carnivores have, similar to terrestrial carnivores, intermediate values of -14‰, ranging from -10‰ in collagen of sea otters to -15‰ in collagen of the common dolphin[238,239]. Thus, also the $\delta^{13}C$ values of marine carnivores compare well with those of their prey. Finally, $\delta^{13}C$ values for the muscle of freshwater fish species ranges from -24 to -13‰, but considerable variation may exist between different lakes.[240] Unfortunately, no $\delta^{13}C$ data are available for terrestrial piscivorous carnivores, but the consistency of the other data suggests that these might be between -24 to -13‰, i.e. comparable to their prey (Figure 7).

Consistent with the data above, Schoeninger et al.[241] showed that European agriculturalists consuming C_3-grasses had much lower $\delta^{13}C$ values in bone collagen (-21 to -19‰) compared to Mesoamerican agriculturalist consuming C_4-corn (-7 to -5‰), while North American and European fisher-gatherers had intermediate values (-15 to -11‰). However, bone collagen proved less reliable to study early *hominin* diets. For that purpose the $^{13}C/^{12}C$ ratio is preferably measured in tooth enamel. To compare collagen $\delta^{13}C$ values to enamel $\delta^{13}C$ values, an additional (8‰) correction has

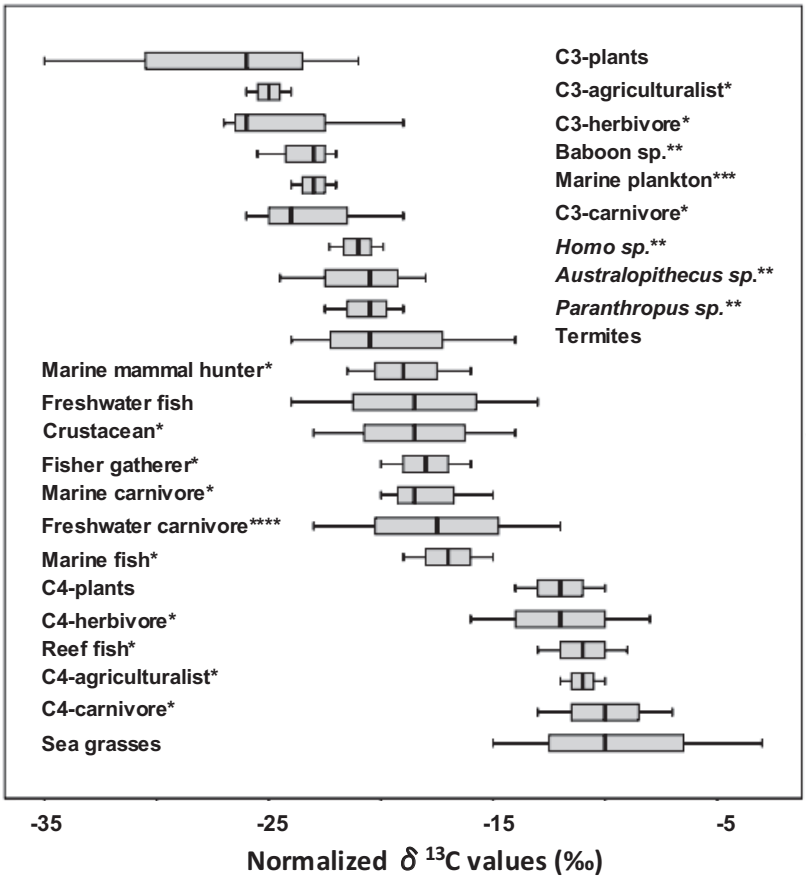

Figure 7. The normalized collagen $\delta^{13}C$ values (mean, range; in ‰) in plankton, crustaceans, sea grasses, C3- and C4-plants; of marine crustaceans, fish and freshwater fish and their respective carnivores, of terrestrial C3- and C4-herbivores and their carnivores; and of human groups in historic and prehistoric times. * Corrected[228] for collagen (-5‰). ** Corrected[237] for enamel (-13‰). *** Arbitrary range of ±1 ‰ due to a lack of data. **** As predicted from other predator-prey relationships and after correction[238] for tropic level (+1‰). Adapted from Ambrose et al.[227,] Sponheimer et al.[230,237,242,246-248], Peters et al.[233] ,Lee-Thorpe et al.[236,243], Kelly[238], Schoeninger et al.[239,241,245], Mbabazi et al.[240] ,Van der Merwe et al.[244]

to be made. Similar to the clear distinctions between bone collagen of modern grazers, browsers and their carnivores, tooth enamel data from fossilized fauna from South-Africa showed similar differences for Plio-Pleistocene C_3-feeders (-11.5‰) and C4-feeders (-0.5‰), with *Australopithecus, Paranthropus* and *Homo* taking intermediate positions (-10 to -4‰)[230,233,236,242], which compared well with the values for contemporaneous felids (-10 to -0.5‰)[230,236,243] (Figure 7). These results clearly demonstrated that a significant portion of the diets of the early *hominins* from Swartkrans, Makapansgat and Sterkfontein derived from C_4-resources. Using these data it was calculated that South-African *Paranthropus* derived 14-47% of its diet from C_4-sources, compared to 5-64% in *Australopithecus* and 20-35% in *Homo.*[244]

A second study in Olduvai showed that the Tanzanian *Paranthropus boisei* derived 77-81% and *Homo* 23-49% from its diet from C_4-resources[244]. The low nutritional value of grasses, and microwear studies (see above), render it unlikely that humans were directly eating grass.[230,242] Analogously, sizeable carnivory of C_4-consuming mammals (such as cane rats, hyraxes or juvenile bovis) was argued to be practically impossible and thus unable to leave a strong C_4-signature.[242] However, at ~1.8 Mya, there were extensive wetlands in the Olduvai area, where a river from the Ngorongoro mountains entered the area, while at 1.5 Mya the Peninj river produced wetlands near Lake Natron.[155] Some researchers investigated the edible plants in a present day wetland (Okavango Delta) and found that the rhizomes and culms of three species of C_4-sedges were edible, the most common one of which is *Cyperus papyrus.*[244] However, it seems unlikely that the C_4-signature in all early *hominins* derived from the consumption of papyrus. It was recently suggested that *P. robustus* and especially *P. boisei* had a diet of primarily C_4-resources, most likely grasses or sedges, from savanna or wetland environments, respectively.[189] Theoretically, a good source of C_4 foods would be a seasonal freshwater wetland with floodplains and perennial marshlands, with an abundance of easy accessible aquatic foods, large aggregations of nesting birds and calving ungulates.[233] Consumption of termites could have contributed to the high C_4-signature observed in hominin fossils (Figure 7), but it seems unlikely that termites could explain values as high as 50% of the diet from C_4[242]. Finally, an enamel C_4-signature of -10 to -4‰ in *hominins,* which translates into a soft tissue signature of -23 to -17‰ and a collagen signature of -18 to -12‰ (see above), might also derive from the consumption of small freshwater aquatic animals/fish, since they compare well with the $\delta^{13}C$ values of -24 to -13‰ for freshwater fish[240] and -18 to -9‰ in collagen of crustaceans and anthropods[239], respectively. Moreover, $\delta^{13}C$ values for hominins are similar to those reported for marine mammal hunters, freshwater fish, crustaceans, fisher gatherers, marine and freshwater carnicores and marine fish (Figure 7).

In agreement with the variability selection hypothesis of Potts[169], which states that large disparities in environmental conditions were responsible for important episodes of adaptive evolution, the wide range in $\delta^{13}C$ values in particularly *Australopithecus* suggests that early hominins utilized a wide range of dietary sources, including C_4-resources. This contrasts with chimpanzees, which, even in the most arid and open areas of their range, are known to consume negligible amounts of C_4-resources, despite their local abundancy. Consequently chimpanzees show very little variability in their $\delta^{13}C$ carbon signature[245,246]. This underscores that even if contemporaneous chimpanzees and early hominins inhabited similar habitats, hominins had broadened their dietary range sufficiently to survive in habitats uninhabitable by chimpanzees. The latter assumption provides an interesting perspective on the recent data, which suggest that C_4-foods were absent in the diet of *Ardipithecus ramidus* at 4.4 Ma. Consequently, it has been proposed that the origins of the introduction of C_4-foods into the hominin diet lie in the period between 3 and 4 Mya.[228]

Limited evidence from the $^{15}N/^{14}N$ ratio

Another stable isotope ratio that has received considerable attention is the nitrogen isotope ($^{15}N/^{14}N$) ratio. A number of food web studies have shown that each step in the food chain is accompanied by 3-4‰ enrichment in $\delta^{15}N$[227,238] and that $\delta^{15}N$ can therefore be useful as a trophic level indicator. Additionally, animals feeding in marine ecosystems have higher values compared to animals feeding on terrestrial resources.[241] For example, North-American and European fisher gatherers and North-American marine mammal hunters and salmon fishers had much higher $\delta^{15}N$ values (+13 to +20‰) compared to agriculturalists (+6 to +12‰). Analyses of phyto- and zooplankton suggest that freshwater organisms have $\delta^{15}N$ values intermediate to terrestrial and marine organisms.[241] $\delta^{15}N$ values are routinely measured in bone collagen, but it was shown that good quality collagen (preserving the original $\delta^{15}N$ value) can, and only under favorable conditions, survive up to a maximum of 200,000 years.[224] This limits $\delta^{15}N$ isotopic studies to Late Pleistocene hominins (see below), but with improved technology, future studies using collagen extracted from tooth enamel may expand their application to early hominins.[237]

Limited evidence from the $^{18}O/^{16}O$ ratio,

A final isotope that might provide information about an animal's diet and thermophysiological adaptations is the oxygen isotope ratio ($^{18}O/^{16}O$). More energy is needed to vaporize $H_2{}^{18}O$ than $H_2{}^{16}O$. When ocean water evaporates and during evapotranspiration, i.e. the sum of evaporation and plant transpiration from the earth's land surface to atmosphere, more of the lighter isotope evaporates as $H_2{}^{16}O$. The ensuing ^{18}O-enrichment of transpiring leaves results in ^{18}O enrichment in typical browsers such as kudu and giraffe who rely less on free drinking water and derive most of their water from the consumption of the ^{18}O-enriched plant water. As the ^{16}O-enriched water vapor in clouds moves inland, some of it condenses as rain, during which more of the heavier isotope (as $H_2{}^{18}O$) rains out, making the $\delta^{18}O$ of coastal rain only slightly less enriched than the original vaporated ocean water, while the $\delta^{18}O$ of the remaining water vapor that eventually comes down is highly negative (i.e. more ^{18}O depleted). Consequently, river water from rain and melting ice is more $\delta^{18}O$ negative than seawater. Roots derive their water from meteoric or underground water that is thus relatively depleted from ^{18}O and so become animals that are consuming these roots.[247,248] Browsers of leaves undergoing evapotranspiration and consumers of roots may thus be expected to have high and low $\delta^{18}O$ values, respectively.

Australopiths showed lower $\delta^{18}O$ values compared to *Paranthropus*, but the meaning of this difference remains uncertain. However, one might argue that *Australopithecus* preferred less arid conditions compared to *Paranthropus* or was more dependent on seasonal drinking water.[230] Low $\delta^{18}O$ were additionally found in primates and suids, which might be linked to frugivory, although this is not supported by the higher ^{18}O values found in *Ardipithecus ramidus* compared to *Australopithicines*.[67,228] Taken together, the use of $\delta^{18}O$ for exploration of ancient human diets is still in its infancy, but might, especially in combination with other isotope ratios, become more

appreciated in the future.

Isotopic data for more recent hominins

It would be of high interest to explore the hominin diet during the last spurt of encephalization between 1.9 million to 100 kya, when brain size tripled in size to volumes between 1,200-1,490 cc for *Homo erectus*, *H. heidelbergensis, H. neanderthalensis* and modern *H. sapiens*.[99] Isotopic data for this period are however absent. Due to limited preservation of collagen beyond 200,000 years, and the near absence of C_4-plants in Europe, these answers will have to come from further studies with tooth enamel in Africa and Asia. So far, there is no isotope evidence for the diet of *Homo* between 1.5 millions years up to 50,000 years ago (kya) *(M.P. Richards, personal communication)*.

Dietary information from more recent humans comes from data on $\delta^{13}C$, supplemented with data on $\delta^{15}N$ and the $^{13}C/^{15}N$ ratio. The ^{15}N-isotope values of bone collagen[241] for differentiation between aquatic and agricultural diets were additionally verified by the study of the sulfur isotope rations ($^{34}S/^{32}S$), since high intakes of marine organisms also result in higher $\delta^{34}S$ values.[249] Combined isotope studies reveal high intakes of animal protein, with substantial portions derived from freshwater fish by Upper and Middle Palaeolithic (40-12 kya) humans in Eurasia, indicating that in some populations about 30% of dietary protein came from marine sources.[249-255] In contrast, isotopic evidence indicates that *Neandertals* were top-level carnivores that obtained most of their dietary protein from large terrestrial herbivores, although even *Neandertals* certainly exploited shellfish such as clams, oysters, mussels and fish on occasion.[249-251] At the onset of the Neolithic period (5,200 years ago), there was a rapid and complete change from aquatic to terrestrial derived proteins among both coastal and inland Britons compared to Mesolithic (9,000-5,200 years ago) British humans[253], which coincides precisely with the local onset of the agricultural revolution in Europe.

Conclusions from isotope studies

The isotope systems that have been studied thus far in hominin bone and teeth provide evidence that early hominins were opportunistic feeders.[256] The spread of C_4-foods in East-Africa and subsequently in the hominin food chain between 3-4 Mya, is in agreement with a niche of early hominins that locates close to the water. This conception is in agreement with the palaeo-environmental evidence. However, many questions still remain unanswered. With regard to the possible niche in the water-land interface, it seems interesting to include aquatic as well as terrestrial piscivorous animals into future studies. The data of combined studies of early hominins and the more recent hominins suggest a gradual increase in dietary animal protein, a part of which may derive from aquatic resources. In the more recent human ancestors, a substantial part of the dietary protein irrefutably derived from marine resources, and this habit was only abandoned in some cases after the introduction of agriculture at the onset of the Neolitic.[253]

5.4 Archeology

The oldest stone tools found so far are dated to 2.6 Mya[257,258] and it has been suggested that these have been used for flesh removal and percussion on long bones for marrow access. From this time onward stone tools were apparently used for defleshing and butchering of large animals. However, again there is a pitfall in putting too much emphasis on the association between stone tools and hunting and butchering of large animals as the sole food source of the human ancestors, especially with regard to brain foods such as long-chain polyunsaturated fatty acids (LCP). As stated by Liem's Paradox; the apparently overwhelming evidence for the consumption of bone marrow, or even brain from cracked skulls, by the findings of cut marks on animal bone may not be evidence for the primary food resources of human ancestors, but only for its fall-back food. Bones, especially long bones, are also better preserved than vegetable material. Moreover, cut marks on bone are easier ascribed to human utilization than any nearby found fossilized fish bones or molluscan shells that only seldomly bear cut marks[259,260] and are often not even examined. Hence, while human remains are nearly always found in the vicinity of water and the fossil record of nearby found fish is extensive,[73,261] the exploitation of aquatic resources is difficult to relate to early humans.[262]

The current review is about the diet that allowed early humans to increase their brain size and thereby become intelligent enough to develop e.g. symbolic thinking and the controlled use of fire. Hunting and/or scavenging is often invoked as important source of LCP, but, as pointed out by Crawford[9], even in the more recent certainly 'hunting' ancestors 'a [scavenged] small brain was not going to go far among the ladies [and children] even if it was still in an edible condition when they [the male hunters] got it back [from the savannah]'[2]; not even in the scenario[263] that we were specialized, as suggested[264], in endurance running. Apart from organ tissue (liver and brain) and bone marrow (whether scavenged or hunted), fish, shellfish and other aquatic foods are also mentioned as rich sources of the nutrients involved in brain expansion.[98,129,265] Therefore, the question arises whether the archeological evidence for human habitation in the land-water-ecosystem only represents facilitated fossilization or indicates the true ecological niche. The following section will focus on comparable evidence for the concurrent exploitation of aquatic resources.

Living in the water-land ecosystem

Because sea levels have risen up to 150 m in the past 17,000 years, a substantial part of the evidence for the exploitation of aquatic resources is hidden below sea level, if not permanently destroyed by the water.[266,267] However, in Kenya, a site in East-Turkana provides solid evidence that at ~1.95 Mya hominins enjoyed carcasses of both terrestrial and aquatic animals including turtles, crocodiles and fish, which were associated with Oldowan artifacts.[260] More ambiguous evidence for the exploitation of freshwater fish, crocodiles, turtles, amphibians and molluscs by *Homo habilis* in the Olduvai Gorge in Tanzania goes back as far as 1.8-1.1 Mya.[266,268] Subsequent tentative evidence from Olduvai Gorge dates the use of similar aquatic resources by *Homo erectus* to 1.1-0.8 Mya.[266,268] Also the Out-of-Africa Diaspora has likely taken place largely via the coastlines,[80] even after the crossing of the Bering

Strait into North America[269] (Figure 2). In Koa Pah Nam, Thailand, 700 kya old piles of freshwater oyster shells were associated with *Homo erectus*.[270,271] In Holon, Israel, freshwater turtles, shells and hippo bones were associated with *Homo erectus* and dated to 500-400 kya.[272] *Homo erectus* fossils associated with seal remains in Mas del Caves (Lunel-Viel, France) were dated to 400 kya.[273]

The archeological evidence for aquatic resource use increases with the appearance of archaic *Homo sapiens*.[266] Although dominated by land mammal bones, 400-200 ky old remains from penguin and cormorants in Duinefontein, South-Africa, were associated with early *Homo*.[274] Shellfish and possibly fish remains, dated 300-230 kya, were associated with the French coastal campsite at Terra Amata,[275,276] while marine shellfish and associated early human remains, dated 186-127 kya, were found in Lazaret, France.[273] Marrean et al.[277] found evidence for the inclusion of marine resources, at 164 kya, in the diet of anatomical modern humans from the Pinnacle Point Caves (South-Africa). At the Eritrea Red Sea coast, Middle Stone Age artefacts on a fossil reef support the view that early humans exploited near-shore marine food resources by at least 125 kya.[278] In several North-African sites, dated to 40-150 kya[266], human remains were associated with shell middens and aquatic resources such as aquatic snails, monk seals, mussels and crabs. Several European sites, dated to 30-125 kya, are comparable to archeological sites that reveal evidence ranging from thick layers of mussels and large heaps of marine shells in Gibraltar[266] to diverse marine shells in Italy,[279] and to a casual description of the presence of marine shells of unknown density in Gruta da Figueira in Portugal[266]. Further evidence for the use of shellfish, sea mammals and flightless birds comes from: Klasies River Mouth (South Africa) dated between 130-55 kya[266,280,281]; from Boegoeberg, where 130-40 ky old shell middens and cormorant bones were associated with *Homo sapiens*[266,282]; from Herolds Bay Cave, where 120-80 ky old shell middens, shellfish, mussels and otter remains were associated with human hearths[266]; from Die Kelders (75-55 kya), where abundant remains of sea mammals, birds and shellfish were found in cave deposits[266,283]; and from Hoedjies Punt (70-60 kya), Sea Harvest (70-60 kya) and Blombos Cave (60-50 kya) for use of shellfish, sea mammals and fish.[266,284] From this period onwards, human settlements are strongly associated with the exploitation of aquatic resources.[266,267,281,285-287] Evidence for more sophisticated fishing by use of barbed bone harpoon points dates back to 90-75 kya in Katanda, Semlike River, Zaire[288,289] and to 70 kya in South Africa.[290] Finally, indications for seafaring are dated to 42-15 kya.[291-293] Possibly, seafaring dates as far back as 800 kya, as indicated by the finding of *Homo erectus* stone tools at the Indonesian island of Flores, which is located on the other side of a deep sea strait.[78,294-298] In general, many archeological sites are found along channels, lake- and seashores[98,265,266,281] and reveal aquatic fauna, such as catfish, crocodile and hippo,[299] but its proves difficult to relate their possible utilization to our early ancestors.

Several events within the time span of the past ~2 million years have been attributed to the increase in brain size and intelligence. The introduction of meat in the hominin diet, which resulted in a higher dietary quality (DQ), has been discussed above. Claims for controlled fire in Olduvai Gorge (Tanzania) and Koobi Fora (Kenya) go as far back as 1.5 Mya.[214,300,301] Evidence for cooking is as old as 250 kya,[300] but possibly dates back to 800 kya,[302] when indications of controlled fire were found to be

present. However, recently it has been concluded that solid evidence for systematic use of fire is only found from 400-300 kya onwards.[303] Evidence that cooking provided increased DQ were recently provided by Wrangham et al.[214] According to archeological evidence, this could only have played an important role since the appearance of *Homo sapiens*[214,300] and Neandertals.[304] Also, the inclusion of aquatic resources as an attributor to human brain evolution has been suggested,[2,98-100,129,265,305-307] but remains a matter of debate.[133,134,267,308]

From hunting-gathering to agriculture

The hunter-gatherer lifestyle continued worldwide for several millions of years and ended quite abruptly with the introduction of agriculture. The first indications for the abandonment of the hunter-gatherer lifestyle towards settlement come from a 23,000 years old fisher-hunter-gatherer's camp at the shore of the Sea of Galilee.[309,310] The associated return from diets containing substantial amounts of protein (from hunting and gathering) back to substantial amounts of carbohydrates is supported by indications for the ground collecting of wild cereals.[311] This was slowly followed by the large scale utilization of cereals starting with the onset of the agricultural revolution some 10,000 years ago.

As indicated above (see 'Biogeochemistry'), there is much controversy about the diet of the earliest humans and until now it is often stated that fishing was only introduced until more recently. From an anthropological perspective this might be true, since certain types (e.g. deepwater) fishing requires advanced techniques.[293] However, from a nutritional point of view, 'fishing' might include anything from collecting sessile shellfish to the seasonal hand capture or clubbing of migrating or spawning fish in very shallow water. Since fresh drinking water is the single most important aquatic resource for humans, hominins probably observed predators and scavengers feeding on aquatic animals. This makes it unlikely that they would not have participated in opportunistic harvesting of the shallow water flora and fauna, such as mollusks, crab, sea urchins, barnacles, shrimp, fish, fish roe or spawn, amphibians, reptiles, small mammals, birds or weeds.[174,266] There are many indications suggesting that the evolution of early *Homo* and its development to *Homo sapiens* did not take place in the "classical" hot, arid and waterless savannah, but occurred in African ecosystems that were notably located in places where the land meets the water (with the land ecosystem possibly consisting of -depending on rainfall- wooded grasslands). Compared with terrestrial hunting and/or scavenging in the savannah, food from this land-water ecosystem is relatively easy to obtain and is rich in the afore mentioned combination of haem-iron, iodine, selenium, vitamins A and D, and long chain ω3-fatty acids.[99,100,265]

In conclusion, there is ample archeological evidence for a shift from consumption of plant towards animal foods. Secondly, although there is an extensive archeological record for aquatic fossils (representing the possible food) in the vicinity of human remains, their co-occurence is usually attributed to the preferential conservation of human remains in the vicinity of water. The current review provides support for the notion that the exploitation of these aquatic resources

by hominins in coastal areas should be the default assumption, unless proven otherwise.[153] For a long time period in hominin evolution, hominins derived large amounts of energy from (terrestrial and aquatic) animal fat and protein. This habit became reversed only by the onset of the Neolithic Revolution in the Middle East starting about 10,000 years ago.

5.5 Anthropology

The hunter-gatherer diet

The *Homo* genus has been on earth for at least 2.4 My[312] and for over 99% of this period they lived as hunter-gatherers.[313] Surprisingly, very little information is available on the macro- and micronutrient compositions of their diet in this extended and important period of human evolution.[34,147] Since the onset of agriculture, about 10 kya, agriculturalists and nomadic pastoralists have been expanding at the expense of hunter-gatherers,[313] with agricultural densities increasing by a factor 10-1,000 compared to the highest hunter densities. For this reason, present day hunter-gatherers are often found in marginal environments, unattractive for crop cultivation or animal husbandry.

In order to study the original hunter-gatherer way of life, it is appropriate to aim at the few hunter-gatherers communities living in the richer environments that bear closer resemblance to those in which the evolution of the genus *Homo* probably took place. Most studies on hunter-gatherers and their diets are, however, performed by anthropologists,[314] whose primary interests different from those of nutritionists. Anthropologists would for example conclude that 'fishing was so unimportant as to be a type of food collection,[315] or consider collecting both small land fauna and shellfish[313] as part of 'gathering', whereas from a nutritional point of view considerable differences exist in caloric density, macro- and micronutrient composition between plants, terrestrial and aquatic animal foods.

Hunting vs. gathering

Studies on food procurement of present day hunter-gatherer societies show, in terms of energy gain versus expenditure, the advantage of hunting compared with plant foraging.[191] Nevertheless, three distinct studies[41,147,313] showed that hunting makes up only ~35% of the subsistence base for worldwide hunter-gatherers, independent of latitude or environment. However, collection of small land fauna and shellfish was included as gathering in these studies. While gathering evidently played an important role over the whole of human evolution; hunting, although introduced later, coincided with 'a major leap for mankind' and has ever since played the most dominating cultural role. While hunting may have overtaken gathering in cultural importance, gathering continued to play a very important nutritional role, because: i) gathering still contributes about 65% to the subsistence base, ii) many micronutrients derive only from plant sources, iii) gathering of e.g. shellfish provides a substantial amount of LCP and other nutrients essential for brain development, and iv) gathering plays an important cultural role since women, children and grandparents can participate.[56,57,316]

Contrary to common belief, hunting in present day hunter-gatherers is still not very successful:

the probability for a kill in !Kung bushmen is only 23%[313] and the subsistence of Hadza, as described by Marlowe[41] and Woodburn[144], is composed for 75-80% of plant foods. Conversely, studies of North-American hunting-gathering societies describe the dietary role of shellfish as similar to 'bread and butter', being the staple food[317] in these societies. The anthropological remark[313] that for many studied hunter-gatherer-tribes 'fishing was only a type of food collection' also adds to the notion that the collection of aquatic foods might have preceded scavenging and hunting. Collecting aquatic foods is still daily practice in eastern Africa and picking up, clubbing or spearing stranded aquatic-animals seems much easier and safer than either scavenging or hunting game on the Serengeti plains.

We conclude that gathering plays, and most likely always played, the major role in food procurement of humans. Although hunting doubtlessly leaves the most prominent signature in the archaeological record, gathering of vegetables and the collection of animal, notably aquatic, resources (regardless of whether their collection is considered as either hunting or gathering); seems much easier compared to hunting on the hot and arid savannah. We suggest that it seems fair to consider these types of foods as an important part of the human diet, unless proven otherwise.[153] Conversely, while hunting might have played a much more important role at higher latitudes, dietary resources in these ecosystems are rich in ω3-fatty acids (e.g. fatty fish and large aquatic mammals), while the hominin invasion of these biomes occurred only after the development of more developed hunting skills.

5.6 (Patho)physiology

Brain selective nutrients

Nutrients and other environmental factors are increasingly recognized to influence epigenetic marks,[318-322] either directly or indirectly via many bodily sensors. Food from the diverse East-African aquatic ecosystems is rich in haem-iron, iodine, selenium, vitamins A and D, and ω3-fatty acids from both vegetable origin and fish.[100] All of these nutrients seem to act at the crossroads of metabolism and inflammation.[24] For example, peroxisome proliferator receptors (PPARs)[323,324] are lipid-driven nuclear receptors with key cellular functions in metabolism and inflammation.[26] Thyroid hormone receptors (TR),[325] the vitamin D receptor (VDR),[326] the retinoid X receptors (RXR) and the retinoic acid receptors (RAR)[327] are other examples of nuclear transcription factors that serve functions as ligand-driven sensors. The iodine and selenium-dependent hormone triiodothyronine (T_3)[328-330] is a ligand of TR,[325] many fatty acids and their derivatives are ligands of PPARs,[331] the vitamin D derived 1,25-dihydroxyvitamin D hormone is a ligand of the VDR,[332] 9-cis retinoic acid and the fish oil fatty acid docosahexaenoic acid (DHA) are ligands of RXR,[327] while RAR interacts with vitamin A (retinol) and many of its derivatives such as all-trans retinoic acid, retinal and retinyl acetate.[327] The ligated nuclear transcription factors usually do not support transcription by themselves, but need to homodimerize or heterodimerize notably with RXR to facilitate gene transcription. Examples of the latter are TR/RXR, PPAR/RXR, VDR/RXR, and RAR/RXR. It has become clear that their modes of action

illustrate the need of balance between e.g. iodine, selenium, fish oil fatty acids and vitamins A and D; a balance that is notably found in the land-water ecosystem.

Deficiencies of the above 'brain selective nutrients' are among the most widely encountered in the current world population.[100,333] While iodide is added to table salt in many countries, margarines and milk have become popular food products for fortification with vitamins A and D. After discussing some general health differences between traditionally living people and those living in westernized countries, we focus on the importance of LCP and notably those of the ω3-series, as examples of the above mentioned nutrients that are especially abundant in the land-water ecosystem,

Hunter-gatherer vs. 'Western' physiology

There are many differences in health indicators between traditionally living people and those living in Western societies. For instance, primary and secondary intervention trials with statins indicate lowest coronary heart disease risk at an LDL cholesterol of 50-70 mg/dL (1.3-1.8 mmol/L), which is consistent with levels encountered in primates in the wild and hunter-gatherer populations with few deaths from cardiovascular disease.[334-338] Another example of the healthy lifestyle of present day hunter-gatherers comes from the observed 'insulinopenia' or 'impaired insulin secretion' following an oral glucose tolerance test (**Figure 8)** in Central African Pygmies and Kalahri Bushmen,[339,340] respectively. As opposed to the 'impairments' noted by these authors, it may also be argued that these researchers were actually witnessing an insulin sensitivity that has become sporadic in Western countries as a consequence of the decrease in physical activity and fitness, increase in fat mass and as a result of the quantity and quality of the foods consumed.[36,132,341] The current consensus is that 'fat is bad' and especially saturated fats have become associated with cardiovascular disease.[342-344] However, traditional Maasai consumed diets high in protein and fat (milk and meat) and low in carbohydrates.[345,346] They had high intakes of saturated fat and cholesterol, showed extensive atherosclerosis with lipid infiltration and fibrous changes, but had very few complicated lesions, and were virtually devoid of cardiovascular disease.[347] The average total- and LDL-cholesterol in these societies was low and did not increase with age.[347] Finally, the physical fitness of people in such traditional societies, such as the Maasai, is often remarkable.[336]

In Kalahari Desert Bushmen and Central African Pygmies, observers could not find any case of high blood pressure and blood pressure did not increase with age.[334,348] Dental surveys of Kalahari Bushmen[349] and other hunter-gatherers[49] showed a remarkable absence of caries. The absence was explained by the repetitive annual abstinence of fermentable sugars in their diet, with a consequent inability to build a cariogenic oral lactobacillus flora.[349] Inhabitants of Kitava (Trobriand Islands, Papua New Guinea) have high intakes (70 en%) of carbohydrates from yams, high intakes of saturated fatty acids from coconuts, and a high fish intake.[44,45,350] Although both high intakes of carbohydrates and saturated fat have been related to the metabolic syndrome and cardiovascular disease, these traditional Kitavians do not show symptoms of the metabolic syndrome and are virtually free from the Western diseases that ensue from it.

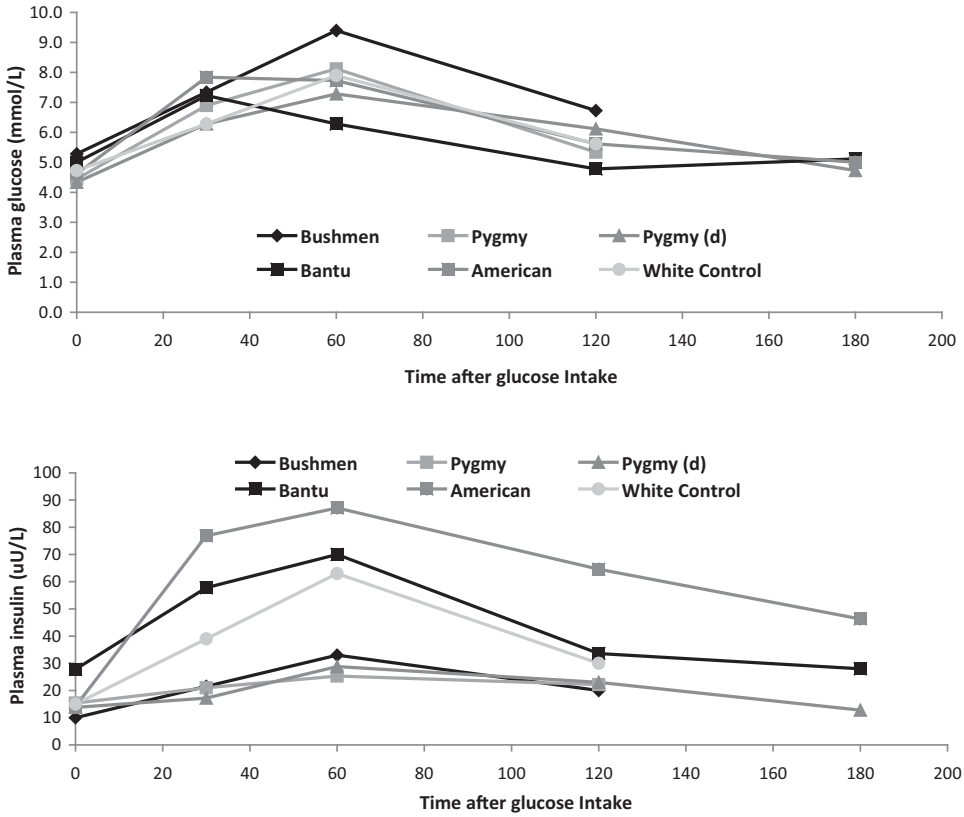

Figure 8. 'Abnormal' insulin response but normal glucose response after oral glucose tolerance test in African Bushmen and Pygmy, compared to Western controls. **A:** Plasma glucose response after a 50g (Bushmen and white controls) and 100g (Pygmy, Bantu and American control) oral glucose load. (d) indicates two weeks daily supplementation of 150g carbohydrates before testing. Of notion is that Bushmen and Pygmy have significant lower body weights as compared to Bantu and white/American controls (average weight Bushmen/Pygmy males 46 kg, females 38 kg; controls 65 kg) while each group received the same unadjusted glucose loading dose of 50g or 100g glucose. **B:** The so called 'abnormal' insulin response or 'impaired' insulin secretion as observed in both Bushmen and Pygmy.[339,340]

Evidence based medicine as applied to LCP in cardiovascular disease and depression

Despite some compelling examples of the healthy lifestyles of traditional populations, current dietary recommendations derive preferably from randomized clinical trials (RCTs) with single nutrients and preferably hard endpoints.[351] This approach clearly oversimplifies the effects of dietary nutrients,[352] since neither macronutrients, nor micronutrients, are consumed in isolation and their effects may be the result of a complex web of interactions between all the nutrients present in the biological systems that we consume, such as a banana or a fish.

The current recommendations from many Nutritional Boards for a daily intake of 450 mg EPA+DHA in adults derives from epidemiological data that demonstrated a negative association of fish consumption with coronary heart disease[353-356] that has subsequently become supported

by landmark trials with ALA[357] and fish oil[358-360] in cardiovascular disease. However, not all trials in cardiovascular disease have been positive.[361] In addition, a negative association was observed for fish consumption and depression[362-364] and for homicide mortality.[365] The causality of these relations was supported by some, but not all, trials with fish oil in depression,[366-370] while a recent meta-analysis demonstrated the beneficial effect of eicosapentaenoic acid (EPA) supplements with ≥60% EPA of total EPA+DHA in a dose range of 200-2,200 mg/day of EPA in excess of DHA.[371]

The influence of polymorphisms in the genome is increasingly recognized, but seldom interpreted in an evolutionary context. As argued above, most polymorphisms were already amongst us when *Homo sapiens* emerged, some 200,000 years ago, while that also holds true for most, if not all, currently identified 'disease susceptibility genes' that are usually abundant but confer low-risk.[372] A loss-of-function mutation in a specific biosynthetic pathway might be an evolutionary advantage if the specific end-product has been a consistent part of the diet, such as is probably applicable to all vitamins, e.g. vitamin C.[373,374] Applied to our LCP status, it is nowadays well established that all humans synthesize DHA with difficulty.[375,376] Analogously, the recently discovered polymorphisms of fatty acid desaturases 1 (FADS1; also named delta-5 desaturase) and FADS2 (delta-6 desaturase) with lower activities in their conversion of the parent EFA to LCP suggest that from at least the time of their appearance, the dietary intakes of AA, EPA and DHA have been of sufficient magnitude to balance the LCPω3/LCPω6 ratio[377,378] to maintain good health.

LCP benefits in pregnancy and early life

Another indication for the importance of LCP comes from the higher LCP contents in the fetal circulation compared to the maternal circulation, a process named biomagnification,[379-381] which occurs at the expense of the maternal LCP status.[382,383] The decreasing maternal LCPω3 status during pregnancy in Western countries is associated with postpartum depression,[362,363] although intervention studies with LCP in postpartum depression have been negative so far.[369,370,384,385] However, a positive effect was seen for LCPω3 supplementation on depression during pregnancy[386] and it has been advocated to start supplementation earlier in pregnancy and with higher dosages.[387]

Maternal LCP intakes have also been related to infant health. AA and DHA in premature and low birth weight infants correlated positively with anthropometrics, AA to increased birth weight[388] and DHA to prolonged gestation.[389-391] Studies with supplementation of DHA during pregnancy yielded e.g. evidence for: i) the maturation of the brain, visual system and retina of the newborn at 2.5 and 4 months, but not at 6 months[392-396]; ii) increased problem solving at 9 months but no difference in memory[397]; and iii) superior eye-hand coordination at 2.5 years[398] and higher IQ at 4 years,[399] but not at 7 years of age.[400] In contrast to the inconclusive human studies, animal studies and combined human and animal studies showed abnormal behavior together with disturbed cognition at lower brain DHA levels.[401] The importance of dietary AA during pregnancy seems less pronounced, but a positive association between umbilical AA and neonatal neurological development[388] and a lower venous AA for those with slightly abnormal neurological development[402] has been shown. A

reduced DHA status in the brain is associated with a mildly increased AA status,[403] which is in its turn associated with low-grade inflammation.[404]

Infant health starts with maternal health, thus dietary recommendations issued for pregnant women indirectly also apply to their infants. The recommendation for adults to consume 450 mg DHA+EPA/day translates into a DHA composition in breast milk of about 0.79%.[405] However, current recommendations for the composition of infant formulae derive mainly from the range of human milk FA compositions as observed in Western countries, which on their turn derive from women with recorded intakes below the 450 mg recommended daily intake of EPA+DHA.[406,407]

The same paradox holds for other fatty acids (FA) in breast milk. For instance, there are few recommendations for the medium chain saturated FA (MCSAFA) content of human milk. High MCSAFA contents in some traditional societies derive from their high intakes of 12:0 and 14:0 from coconuts.[408] Conversely, the high MCSAFA contents in Western populations are primarily influenced by the maternal carbohydrate intakes,[409] since the mammary gland has the unique ability to convert glucose into MCSAFA (6:0-14:0), mainly lauric (12:0) and myristic (14:0) acids. However, women with regular consumption of coconuts have a much higher 12:0/14:0 ratio compared to women with high carbohydrate intakes. Both MCSAFA are readily absorbed in the gastrointestinal tract, while antiviral as well as antibacterial properties have been attributed to some MCSAFA, but mainly to 12:0.[410,411]

The PUFA content of Western milk has increased over the last decades.[412,413] While the human milk LA content in the US increased by at least 250%, its DHA content decreased by almost 50% %[413]. The (from an evolutionary point of view) abnormally high LA intake is, despite a lack of evidence,[414] advocated for cardiovascular health.[415] The resulting high LA status is likely to interfere with both the incorporation of AA and DHA into phospholipids and also inhibits their synthesis from their parent EFA.[416] Major differences are noted in the comparison of the human milk fatty acid compositions of Western mothers compared to some traditional African women,[408,417] with unknown consequences for infant health or the occurrence of disease at adult age (i.e. the 'Barker hypothesis').[16,418] It has been proposed that the high concentrations of EPA, DHA as well as AA in human milk, such as described for many fish consuming societies[408,419,420] might be a more appropriate reflection of the Palaeolithic breast milk composition and may therefore constitute a better reference for infant formulae than do Western human milks.[421]

The influence of environment

It is estimated that 70% of all cases of stroke and colon cancer, 80% of all cardiovascular diseases and 90% of all cases of type 2 diabetes mellitus have been caused by lifestyle and could have been prevented by paying more attention to modifiable behavior factors, including specific aspects of diet, overweight, inactivity and smoking.[422] The mismatch between the human diet and the Palaeolithic genome might therefore be responsible for many typically Western diseases. In addition to the evidence from many other disciplines, evidence from (patho)physiology and epidemiology

adds to the notion that a great deal of information on healthy diets might derive from the study of the diets of the early human ancestors. The metabolic syndrome, characterized by impaired insulin sensitivity, is at the center of many diseases of civilization. High intakes of refined carbohydrates as well as low intakes of LCP have been implicated in the development of insulin resistance. As such, low carbohydrate intakes[423,424] and high LCP[8,34,425,426] intakes by the early human ancestor might explain in part the low incidence of diseases of civilization in current hunter-gatherer societies. The available evidence from pathophysiology and epidemiology supports the hypothesis that the land-water ecosystem contributed important and indispensable nutrients to evolving hominins.

5.7 Dietary reconstruction of the nutrients available in eastern Africa

The debate on the ecological niche of human ancestors is unlikely to reach a consensus shortly. The millions of years of human evolution concurred with marked and abrupt climatic changes, which renders a single ecological niche of human ancestry unlikely. However, it is at the same time clear that in a short period of time humans have made tremendous changes in their lifestyle, their diet included, that lie at the basis of the diseases of Western civilization. This prompted various investigators to reconstruct the possible compositions of diets that could have been consumed by our Palaeolithic ancestors. Their studies are e.g. based on the plausibility that prior to the agricultural revolution, when humans lived as hunter-gatherers, cereals were no appreciable part of the diet and that wild animals living in the eastern African savannah and in eastern African aquatic ecosystems have different fatty acid compositions compared to the domesticated animals that have now become staple foods. For example, the lean savannah animals that inhabit the eastern African plains have much lower fat contents, and the available fat is much more enriched in PUFA[427]. Similarly, high latitude (fatty) fish have much higher EPA and DHA contents, but lower AA contents compared to low latitude (lean) fish from tropical waters.[428-431]

Eaton and Konner[8] where the first to use this approach in reconstructing a Palaeolithic diet; their pioneer study was published in the New England Journal of Medicine in 1985. The authors estimated that late Palaeolithic humans consumed diets containing 35% meat and 65% vegetable foods, containing 34 energy% (en%) from protein, 45 en% from carbohydrate and 21 en% from fat, while the ratio between polyunsaturated and saturated fat equaled 1.41 and their fiber intake amounted to 46 g/d.[8] These outcomes contrast with the average American diet at date, that consisted of 12 en% protein, 46 en% carbohydrate and 42 en% fat, with a polyunsaturated/saturated fat (P/S) ratio of 0.44 and a fiber intake of 20 g/d. After twenty-five years of additional study, they confirmed their previous findings by estimating that the Palaeolithic diet provided 25-30 en% protein, 35-40 en% carbohydrate and 20-35 en% fat,[432] while the P/S ratio was 1.40[433]. Moreover, they concluded that "it has become clear since our initial publications that marine, lacustrine, and riverine species were important sources of animal flesh during the evolution of modern *Homo sapiens*, and may have played a role in the evolution of brain ontogeny."[432] In addition to the earlier studies, they also estimated the vitamin and mineral composition of a Palaeolithic diet, showing higher contents of

folate, riboflavin, thiamin, vitamins A and E, calcium, magnesium, phosphorus, zinc, and notably ascorbate, vitamin D (sunlight), copper, iron, manganese and potassium, while the Palaeolithic diet contained much lower sodium compared to contemporary U.S. intakes and recommendations.[432-434] In a subsequent study they estimated that in different ancient hunting and gathering populations, fatty acid intakes would have ranged from 5.19-20.6 g LA/d, 0.26-4.8 g AA/d, 3.45-25.2 g ALA/d and 0.03-1.52 g DHA/d, which contrasted with the much higher LA (22.5 g/d) and lower ALA (1.2 g/d), AA (0.6 g/d) and DHA (0.08 g/d) intakes as observed in current Western populations.[34]

In a meticulous analysis of world wide hunter-gatherer diets, Cordain et al.[147,435] estimated that the most plausible percentages total energy from dietary macronutrients would be 19-35 en% from protein, 22-40 en% from carbohydrate and 28-58 en% from fat, which reflects a markedly higher contribution of dietary fat, a similar amount of protein, but a lower contribution of carbohydrates, compared to Eaton and Konner's earlier estimates.[8,433] The main differences were explained by the assumption that, wherever it was ecologically possible, hunter-gatherers would have consumed 45-65% of total energy from animal foods,[147] while in the earlier estimations[8,433] only 35% derived from animal foods. These higher animal food intakes were explained by their inclusion of both worldwide hunting and fishing hunting-gathering societies into their new calculation models,[147] also including mounted and arctic hunters. Those latter possibilities, however, seem insignificant with regard to early human evolution, which explains why they seem to overestimate the amount of the diet that is derived from animal foods. For example, Marlowe[41] estimated that in a warm-climate sample about 53% of the diet derives from gathering, 26% from hunting and 21% from fishing (i.e. about 47% from hunting).

To subsequently investigate the nutrient compositions of such diets, fish consumption was incorporated as a separate variable to plant and meat consumption in the earlier models, since aquatic and terrestrial animals have markedly different fatty acid compositions. In this most recent analysis[436] 3,000 kcal Palaeolithic diets were investigated with plant/animal food intake ratios ranging from 70/30 to 30/70 en%/en% under the conditions of four different foraging strategies in which the animal part ranged from exclusive meat consumption including the selective consumption of energy- and LCP-rich fat from bone marrow and brain, respectively,[426] to the consumption of an entirely aquatic diet in an eastern African water-land ecosystem.[437,438] It was found that that the energy intakes from the macronutrients were: 25-29 en% (range 8-35) from protein, 39-40 en% (19-48) from carbohydrate and 30-39 en% (range 20-72) from fat. Dietary LA ranged from 1.7-6.2 en%/day, AA from 1.15-10.7 g/day, ALA from 2.1-5.8 en%/day and EPA+DHA intakes from 0.87-28.3 g/day.[436] From these data, despite its wide range in outcomes, it can again be concluded that there are substantial differences with respect to the average composition of the current Western diet, notably because of its higher proportions of carbohydrates and LA, and its much lower protein and ALA and LCP contents. It became also conceivable that ancestors living in the East African water-land ecosystem had daily intakes of gram amounts of EPA+DHA. As such, these LCPω3 intakes were comparable with those of the traditionally living Eskimos in Greenland, who because of their low

cardiovascular disease risk[353,354] initiated the current interest in the role of LCPω3 in both primary and secondary prevention of cardiovascular disease. In addition to these ω3-fatty acids, the water-land ecosystem is also a rich source of haem-iron, iodine, selenium and the vitamins A and D[100], which have important functions and interactions in gene transcription and metabolism.[24,26,439]

6. Dietary changes since the agricultural revolution

Whatever the specific composition and wide range of early hunter-gatherer diets, the current consensus is that our diet has changed markedly from the time of large-scale utilization of cereals and animal domestication (i.e. the agricultural revolution) starting some 10,000 years ago. Contrary to earlier belief, the advent of agriculture coincided with an overall decline in nutrition and general health, but at the same time provided an evolutionary advantage since it increased birth rates and thereby promoted net population growth.[49,50]

While the decline of nutritional quality and general health started with the onset of the agricultural revolution, these processes became even more pronounced with the advent of the industrial revolution some 100-200 years ago.[9,11,132] Among the many dietary and lifestyle changes (**Figure 9**) are: a grossly decreased ω3/ω6 fatty acid ratio, the combined high intakes of saturated fatty acids (SAFA) and carbohydrates,[440-442] the introduction of industrially produced *trans* fatty acids, reduced intakes of LCPω3 and LCPω6 fatty acids, reduced exposure to sunlight, low intakes of vitamins D and K, disbalanced antioxidant status and high intakes of carbohydrates with high glycemic indices and loads, such as sucrose and industrially produced high fructose corn syrup.[36,132,443,444] Many of these changes act in concert, which points at the serious limitations of conclusions from contemporary investigations that study the many nutrients in isolation and form the basis of modern nutritional guidelines. An example is the interaction of dietary carbohydrates with saturated fatty acids.[440-442,445]

7. Potential benefits of a Palaeolithic diet

Evidence for the beneficial effects of Palaeolithic diets may derive from their influence on weight reduction and classical CAD risk factors. In an uncontrolled study with healthy adults, Osterdahl et al.[446] showed a decrease in weight, BMI and waist circumference after 3 weeks *ad libitum* consumption of a Palaeolithic-like diet (i.e. 1,584 kcal/day; carbohydrate 40, protein 24, fat 36 en%), compared with their baseline usual diet (2,478 kcal/day; carbohydrate 54, protein 14, fat 30 en%). Additionally, they showed favorable effects on systolic blood pressure and plasminogen activator inhibitor-1. Jonsson et al.[447] performed a cross-over study of 2*3 months in type 2 diabetic patients receiving a Palaeolithic diet (1,581 kcal/day, carbohydrate 32, protein 24, fat 39 en%) or a diabetes diet (1,878 kcal/day, carbohydrate 42, protein 20, fat 34 en%). They showed a reduction of body weight, BMI and waist circumference and lower HbA_{1c}, triglycerides, diastolic blood pressure, and higher HDL-cholesterol after consumption of the Palaeolithic diet.

In a randomized trial in patients with ischemic heart disease plus glucose intolerance or type 2 diabetes, Lindeberg et al.[448] showed a reduced caloric intake after *ad libitum* consumption of a

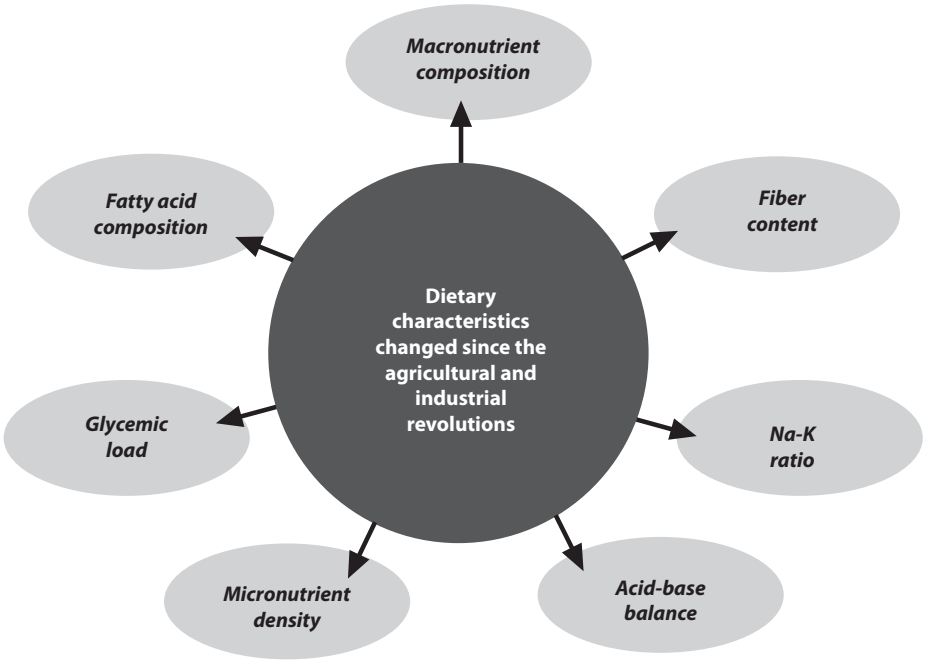

Figure 9. The seven dietary characteristics that have been changed since the agricultural and industrial revolutions. Adapted from Muskiet.[24]

Palaeolithic diet (1,344 kcal/day; carbohydrate 40, protein 28, fat 27 en%) as compared to an *ad libitum* Mediterranean-like Consensus diet (1,795 kcal/day; carbohydrate 52, protein 21, fat 25 en%). They also observed a larger improvement in glucose tolerance in the Palaeolithic diet-group, independent of decreased waist circumference. The most convincing evidence so far derives from an uncontrolled trial[449] showing that 10 days consumption of an isocaloric Palaeolithic diet (2,701 kcal/day; carbohydrate 38, protein 30, fat 32 en%) improved blood pressure, arterial distensibility, insulin sensitivity and total-, HDL- and LDL-cholesterol in healthy sedentary humans, when compared with their baseline usual diet (2,372 kcal/day, carbohydrate 44, protein 18, fat 38 en%). Importantly, there were no changes in energy intakes, activity levels and body weight, which indicates that the improved CAD risk profile was unrelated to weight reduction or well other known determinants.

8. Conclusions

The optimal nutrient combination to support good health can be expected to reflect a certain balance. This balance is present in the foods that were consumed by Palaeolithic and possibly by pre-Palaeolithic ancestors, because it is this balance on which the human genome has evolved. This genome has been shaped by millions of years of evolution, during which it adapted to the conditions of existence, including the diet. There are ample indications from many disciplines that the human ancestors evolved in a water-land interface that provided food from both terrestrial and

aquatic resources. For instance, the availability of both LCPω6 and LCPω3 from the aquatic food chain was one of the many factors that provided early humans the unique combination of brain selective nutrients for brain growth[2]. The recent deviation from this Palaeolithic diet and lifestyle in general might be at the basis of many, if not all, current diseases of civilization. Detailed studies with respect to the health effects of the diets of these earlier ancestors are therefore warranted.

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CHAPTER 2

Estimated macronutrient and fatty acid intakes from an East African Paleolithic diet

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ABSTRACT

Background: Our genome slowly adapts to changing conditions of existence. Many diseases of civilization result from mismatches between our Paleolithic genome and the rapidly changing environment, including our diet.

Objective: Reconstruction of multiple Paleolithic diets to estimate the ranges of nutrient-intakes upon which humans evolved.

Design: A database of, predominantly East-African, plant and animal-foods (meat/fish) was employed to model multiple Paleolithic diets, using two pathophysiological constraints [i.e. protein<35 energy% (en%); linoleic acid (LA)>1.0 en%], at known hunter-gatherer plant/animal-food intake ratios (range 70/30-30/70 en%/en%). We investigated selective and non-selective savannah, savannah/aquatic and aquatic hunter-gatherer/scavenger foraging-strategies.

Results: We found (range of medians in en%) intakes of: moderate-high protein[25-29] moderate-high fat[30-39] and moderate carbohydrates.[39-40] The fatty acid (FA) composition was: saturated-FA (11.4-12.0), monounsaturated-FA (5.6-18.5) and polyunsaturated-FA (8.6-15.2). The latter was high in alpha-linolenic acid (ALA) (3.7-4.7 en%), low in LA (2.3-3.6 en%), and high in long-chain polyunsaturated-FA (LCP; 4.75-25.8 g/day), LCPω3 (2.26-17.0 g/day), LCPω6 (2.54-8.84 g/day), ALA/LA-ratio (1.12-1.64 g/g) and LCPω3/LCPω6-ratio (0.84-1.92 g/g). Consistent with the wide range of employed variables, nutrient intakes showed wide ranges.

Conclusions: Compared to Western diets, Paleolithic diets contained consistently higher protein and LCP, and lower LA. These are likely to contribute to the known beneficial effects of Paleolithic-like diets, e.g. through increased satiety/satiation. Disparities between Paleolithic, contemporary and recommended intakes might be important factors underlying the etiology of common Western diseases. Data on Paleolithic diets and lifestyle, rather than the investigation of single nutrients, might be useful for the rational design of clinical trials.

INTRODUCTION

Our genome is the product of millions of years of evolution in which it slowly adapted to ensure reproductive success under the environmental selective pressures imposed upon our species.[1] Evolutionary medicine predicts that many complex degenerative diseases originate from unfavorable changes in our environment that, in the light of our long generation time, are too rapid to cause appropriate adaptation of our slowly adapting genome.[2] Such genetic adaptations are also unlikely to occur, since these unfavorable changes exert little selection pressure. That is, they do not cause death prior to reproductive age, but rather reduce years in health at the end of the life cycle.[1,3] Our nevertheless increased life expectancy, originates mostly from technological achievements (e.g. the introduction of public health sanitation, the prevention of (childhood) infections, famine, homicide and tribal warfare),[4] which diminish the influence of certain unfavorable conditions of existence. Since the agricultural revolution (some 10,000 years ago) and notably since the industrial revolution (some 200 years ago) we introduced numerous unfavorable changes into our environment and lifestyle. These factors include changes in diet, physical activity, stress, sleep duration, environmental pollution among others. Important dietary and environmental changes, especially in affluent countries, that may adversely affect health and well being include: a decreased ω3/ω6 fatty acid ratio, the combining of high intakes of saturated fatty acids (SAFA) and carbohydrates,[5] introduction of industrially produced *trans* fatty acids, reduced exposure to sunlight, lower intakes of vitamins D and K, imbalanced intake of antioxidants, high intakes of carbohydrates with high glycemic indices and loads, and little dietary fiber. Together with a sedentary lifestyle, these dietary alterations gave rise to an unprecedented body composition characterized by increased fat mass and sarcopenia.[6] These culturally driven environmental changes have exceeded the flexibility of our epigenotype to adapt and have resulted in a maladapted phenotype, primarily after reproductive age.

It has been hypothesized[1] that the range of optimal nutrient combinations to support good health are present in the foods that were consumed by our Paleolithic ancestors living from 2.5 million to 10.000 years ago. Their diets and environment represent the selective pressures under which our genome evolved. The fish oil fatty acids EPA and DHA (and their derivatives), vitamin D (1,25-dihydroxyvitamin D) and vitamin A (retinoic acid) are examples of nutrients that act in concert, while each of these have multiple actions.[7,8] Consequently, criteria for establishing optimum nutrient intakes via randomized controlled trials (RCT) with single nutrients at a given dose and with a single endpoint have serious limitations. They are usually based upon poorly researched dose-response relationships and typically ignore many possible nutrient interactions and metabolic interrelationships. For instance the adequate intake of LA to prevent LA-deficiency is dependent upon concurrent intakes of e.g. alpha-linolenic acid (ALA), γ-linoleic acid and arachidonic acid (AA). Consequently, the nutritional balance on which our genome evolved is virtually impossible to determine by using the reigning paradigm of 'evidence based medicine' with RCTs. Nutritional research rather needs an organizational template that focuses upon optimal homeostasis. This template may be obtained from the reconstruction of Paleolithic diets. Disparity between the range

of nutrients found in the current Western diet and reconstructed Paleolithic diets, will provide direction for guiding future dietary interventions.

The composition of Paleolithic diets may be derived from many disciplines, including biology, archeology, anthropology, comparative anatomy, genetics, food science and (patho)physiology. For instance, the sites at which fossil remains of our hominin ancestors have been discovered suggest that the evolution to anatomically modern humans took place on a long chain ω3-fatty acid rich diet in an East-African water-land ecosystem.[9-12] Additionally, the last Out-of-Africa Diaspora, starting some 100,000 years ago, largely took place via the coastal lines,[13] including crossing into the Americas via the Bering Strait.[14] Compared with hunting in the savanna, food from these ecosystems is relatively easy to obtain and rich in heme-iron, iodine, zinc, copper, selenium, vitamins A and D, and ω3-fatty acids from both vegetables and fish, which are collectively referred to as 'brain-selective nutrients'.[15,16] Epidemiological data as well as landmark trials with ω3-fatty acids or fish consumption demonstrated favorable outcomes for coronary heart disease (CAD),[17-19] (postpartum) depression,[20,21] homicide mortality[21] and neurodevelopment.[22] The importance of dietary LCP is also supported by our low capability to synthesize LCP during the entire life cycle,[23] suggesting that ancestral human intakes of AA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were sufficient for survival and reproductive success. The recently discovered polymorphisms[24] of fatty acid desaturases 1 (FADS1; also named delta-5 desaturase) and FADS 2 (delta-6 desaturase) with lower activities in their conversion of ALA and LA to LCP add to the notion that at the time of the first occurrence of these mutations, dietary LCP intakes compensated for the concomitantly lower LCP synthesis.

Eaton et al.[1,3,25,26] were the first to reconstruct a Paleolithic diet. They assumed a savannah-type diet with non-selective consumption of animals, implying that all available organs were consumed. This model was further refined by Cordain et al.,[27,28] who assumed a savannah diet with multiple subsistence ratios and an 'optimal foraging' strategy. 'Optimal foraging' assumes the preferred consumption of energy dense foods[28-30] (e.g. by the selective consumption of plants with high fat percentages and the selective consumption of energy dense animal organs, such as brain and bone marrow). Compared to contemporary intakes, both models[26-28] suggested lower carbohydrate and higher intakes of protein and LCP. None of the earlier models separately evaluated food intake from the water-land ecosystem, which is the presumed niche of our ancestors[10-14] and an abundant source of EPA and DHA.

We estimated the dietary macronutrient (carbohydrate, protein, fat) and fatty acid compositions for four foraging strategies ascribed to Paleolithic hunter-gatherer/scavengers who foraged in the savannah (Model 1), a water-land ecosystem (Model 4) and a combination of both (Models 2 and 3). The aim was to determine the composition and range of dietary macronutrients and fatty acids under which the human genome evolved and which likely would support modern day health and well-being. In contrast to Eaton et al.[26] our modeling of the savannah diet assumed selective consumption of organs, while compared to the savanna diet of Cordain et al.,[28] we additionally

varied the contributions from muscle, marrow and brain. Our purely aquatic foraging strategy (Model 4) has not been previously evaluated. For the reconstruction we differentiated between selective (Models 1 and 3) and non-selective (Models 2 and 4) meat consumption. Within each model we varied the plant/animal subsistence ratios, the meat/fish ratios (Models 2 and 3), the fat contents of the consumed plants, meat and fish (Models 2-4) and the muscle/marrow/brain ratios (Models 1 and 3). The final outcome was subjected to certain pathophysiological constraints, since not all dietary combinations were considered to be compatible with health. In evolutionary terms, health may be defined as evolutionary fitness to survive up to reproductive age and beyond for successful reproduction and to take care of the younger (the so called grandmother hypothesis[31,32]), respectively.

METHODS

Background for the models

For the calculation of the average macronutrient and fatty acid intakes we divided the diet into two main components (i.e. plant and animal foods). In contrast to earlier models,[26,28] we subdivided animal food into meat and fish, in which the former was further subdivided into five edible components, namely skeletal muscle, brain, marrow, liver and adipose tissue. Additional organs (e.g. kidney, adrenals, spleen, heart, blood etc.) were considered to be of minor interest. They were not included due to limited data on nutrient compositions and a relatively small contribution to overall weight. Since we aimed at investigating the possible ranges of nutrient intakes from multiple Paleolithic diets, we included a wide, but necessarily possible, range of variables. In all models (see below), the plant/animal food ratios in energy% (en%) were varied from 70/30 to 30/70 en%/en%, which is in the range of the most commonly observed hunter-gatherer subsistence ratios.[26,28] The fat contents by weight (g/100 g edible material; g%) of all plants, meat and fish were varied from 2.5-5.0 g%, 5.0-30 g% and 2.5-10.0 g%, respectively, for which justification is given in each particular section below. The macronutrient and fatty acids contents of edible material were taken from the literature, internet food databases[33] and our own data. Detailed information on energy and fat contents and the fatty acid compositions of the various foods and their literature references are given in **supplementary Table 1**. **Table 1** presents the means, as compiled from these raw data. Using the above approach, we calculated the outcomes of four different models, representing four foraging strategies ascribed to early *Homo* and assuming a daily energy intake of 12,500 kJ.

Description of the models

Model 1 describes a *selective hunter-gatherer/scavenger savannah diet* that is composed of plant and animal foods that are effectively available on the savannah, while the consumption of aquatic food was excluded. The employed hard data are in supplemental Table 1 and Table 1. 'Selectivity' implies that from the animal food only the skeletal muscle, marrow and brain were consumed and thus not the liver or adipose tissue. The intakes were varied between 98-0 en% (skeletal muscle),

Table 1. Mean energy, fat contents and fatty acid compositions of the edible parts of foods available to our Paleolithic ancestors

Food	Origin of the data	Species/ specimen	Energy kJ/100 g	Fat g/100 g	ALA g/100 g	EPA g/100 g	DPA g/100 g	DHA g/100 g	w3 g/100 g	LCPw3 g/100 g
Plants	World	49/49	586	3.9	26.1	0.61	0.15	0.32	34.1	0.94
Fish	Africa	68/68	469	3.5	1.06	5.18	3.76	17.4	28.4	27.1
Muscle	Africa	11/51	452	2.9	4.24	1.34	2.92	0.37	8.87	4.64
Brain	World	7/50	528	9.1	0.20	0.04	0.63	9.26	10.1	9.93
Liver	World	48/54	632	6.7	4.32	0.99	2.81	1.93	10.3	5.80
Bone marrow	World	11/73	2.043	51	1.47	0.08	0.06	0.07	1.64	0.21
Adipose tissue	World	13/68	3.120	84	3.56	0.01	0.07	0.04	2.53	0.09

Food	LA g/100 g	AA g/100 g	w6 g/100 g	LCPw6 g/100 g	LCP g/100 g	SAFA g/100 g	MUFA g/100 g	PUFA g/100 g	LA/AA g/g	(EPA+DHA) /AA g/g
Plants	14.0	0.64	15.8	0.87	1.81	29.2	12.5	42.3	0.54	1.45
Fish	2.19	8.45	16.0	13.8	40.9	37.0	18.6	44.4	2.07	2.67
Muscle	20.6	6.40	28.6	7.17	11.2	39.2	21.6	36.3	4.85	0.27
Brain	0.69	5.74	12.0	11.3	21.3	31.8	27.6	22.1	3.51	1.62
Liver	14.9	9.40	26.2	10.2	16.0	35.3	26.0	35.9	3.44	0.31
Bone marrow	3.18	0.16	3.60	0.23	0.44	23.2	64.5	6.04	2.16	0.94
Adipose tissue	5.41	0.21	5.86	0.22	0.49	51.1	35.2	9.53	1.52	0.25

Energy is in kJ/100 g material, fat is in g/100 g material; fatty acids are in g/100 g fatty acids; fatty acid ratios are in g/g

ALA, α-linolenic acid, 18:3ω3; EPA, eicosapentaenoic acid, 20:5ω3; DPA, docosapentaenoic acid, 22:5ω3; DHA, docosahexaenoic acid, 22:6ω3;

LA, linoleic acid,18:2ω6; AA, arachidonic acid, 20:4ω6; SAFA, sum of all saturated fatty acids (FA); MUFA, sum of all monounsaturated FA;

1-80 en% (bone marrow) and 1-20 en% (brain) of the total meat intake. Model 2 describes a *non-selective hunter-gatherer/scavenger savannah/aquatic diet* that contains plant and animal foods that are available on the savannah and in an aquatic environment. In accordance with Eaton,[26] this model assumes the whole animal carcass was consumed (i.e. non-selective), including most animal organs and also the skin and the head in case of fish. The meat and fish intakes were varied from 100-0 and 0-100 en% of total animal consumption, respectively. For the fish we used the energy, fat and fatty acid contents of East-African lake and marine fish because of their specific fatty acid compositions.[34,35] Model 3 describes a *selective hunter-gatherer/scavenger savannah/aquatic diet*. The model assumes a diet from aquatic resources and the selective scavenging of muscle, bone marrow and brain from savannah animals. The meat and fish intakes were again varied from 100-0 and 0-100 en% of total animal consumption, respectively. In this model we varied the intakes from muscle, bone marrow and brain from 0-50 en% (muscle), 40-80 en% (bone marrow) and 10-20 en% (brain) of total meat intake. The fish and plant fat contents were both set at 5 g% in this model, while the average fat contents of the combined muscle/marrow/brain in meat varied from 10-30 g%. Model 4 describes a *non-selective hunter-gatherer/scavenger aquatic diet* that is composed of plants and fish, while the consumption of meat was not included. Consequently, the fish intakes were 100% of animal foods in all applied subsistence ratios. The fish fat content was varied from 2.5 to 10.0 g%. For the energy, fat and fatty acid content of consumed aquatic foods we only applied data for East African fish species.

Justification for the models

The range of the subsistence ratios applied in our models needed evaluation because of the absence of accurate data on human nutritional (plant/animal en%/en%) subsistence ratios in the Paleolithic. Unfortunately, plant/animal (en/en%) subsistence ratios cannot simply be derived from gathering/hunting subsistence ratios. Anthropological studies that differentiate between gathering and hunting often include gathered plant as well as unimportant[36] and small[37,38] animal foods into 'gathering'. They also report the contribution of gathering as a percentage of subsistence economy rather than a percentage of energy (en%). Secondly, in contrast to common belief, hunting probably played a less dominant role from a nutritional point of view as compared to gathering and on average makes up 35% of the subsistence base for present day worldwide hunter-gatherers, independent of latitude or environment.[27,38] For example, hunting by some surviving hunter-gatherers is still not very successful: the probability for a kill in !Kung bushmen is only 23%[38] and the subsistence of Hadzabe, as described by Woodburn[39] consists of 80% plant foods. In the Paleolithic however, hunting might have been more productive, due to both higher animal biomass and since hunter-gatherers had not been displaced yet into marginal environments, unattractive for crop cultivation or cattle. Consequently, we chose the employed ratios within the range of the most commonly observed hunter-gatherer subsistence ratios.[26,27]

The justification for the employed energy densities comes from the common misconception

that members of present day affluent societies are taller than our ancestors. The average height of Paleolithic humans would have placed them within the tallest 15% of our population.[40] Our former nomadic lifestyle as a hunter-gatherer was characterized by vigorous physical activity and lean body mass in contrast to the present day sedentary lifestyle and worldwide increasing body mass indices. The anatomical features and physical activity of pre-agricultural humans probably demanded a greater caloric intake than necessary for current Western populations. The total energy expenditure of *Homo habilis* was estimated to be 10,000 kJ/day[41] and 8,961 kJ/day for !Kung bushmen,[38] but was probably somewhat higher for early Homo sapiens.[42] Energy intakes should be in concordance with the physical activity level (PAL); that represents the ratio between the variable total energy expenditure (TEE) and the constant resting metabolic rate (RMR). A typical PAL in the Paleolithic would be 1.74, compared to 1.4 for a typical sedentary American and 1.75 as recommended by the WHO.[43] The daily energy expenditure, as physical activity, in the Paleolithic, however was estimated at 5,193 kJ, with a total caloric intake of 12,144 kJ, but sedentary humans consume 8,500 kJ/day, while spending only 2,324 kJ/day on physical activity. Adjustment of the caloric intake to the current PAL, would however inherently imply lower nutrient intakes (i.e. LCP and micronutrients), compared to our Paleolithic ancestors. To preclude underestimation of the Paleolithic nutrient intakes, we therefore employed the daily intake of 12,500 kJ/day.

We employed selective *hunter-gathering/scavenging* (i.e. including brain and bone marrow) in Models 1 and 3, but non-selective *hunter-gathering/scavenging* (i.e. including all edible organs) in Models 2 and 4. Selective organ consumption would increase both the fat (hence the caloric) and the LCP content of the meat (Table 1 and **Table 2**). To elucidate this net effect of selective organ consumption we modeled the influence of increased organ tissue consumption[44-47] on the macronutrient and fatty acid intakes. The exclusion of liver and adipose tissue in Models 1 and 3 comes from the observation that scavenged leftovers from carnivore kills seldom contain energy dense organs such as the liver and adipose tissue.[45] Liver and adipose tissue are the first to be consumed by the obligate carnivore, while the head and bones are the most likely leftovers because of their inaccessibility.[44] Although it has been suggested that, apart from the remaining muscle meat,[44,45,47,48] tool-using hunter-gatherer scavengers[48] could have had selective access to brain and marrow[44-47] for the main period of human evolution; it seems unlikely that brain or marrow would have been as easily accessible for consumption, especially for women and children, as aquatic LCP-rich animal foods. Although both marrow and brain are sizeable, energy dense, organs[45-47] (**Table 3**), a substantial contribution from marrow and brain to any regular diet seems unlikely with regard to their sizes and perishability. The high (80/20 en%/en% from marrow/brain to the total meat en%) was therefore included to investigate one of the possible ranges of nutrient intakes, rather than the most realistic or average. From approximately the middle Stone Age on, after humans became top predators, brain and marrow, but also liver and adipose tissue would have become more frequently accessible (i.e. Model 2). The selective consumption of energy dense organs (fat) is consistent with the optimal foraging theory and their preferential consumption was indeed observed in present

Table 2. Total energy from all macronutrients, and contributing percentages of protein and fat to this total energy content; for plants, meat and fish at different fat contents

	Total energy from all macronutrients (kJ/100 g tissue)				Energy% from protein				Energy % from fat			
		Meat	Meat			Meat	Meat			Meat	Meat	
Fat (g%)	Plants	Non-selective	Selective	Fish	Plants	Non-selective	Selective	Fish	Plants	Non-selective	Selective	Fish
2.5	469			436	13	79		77	19	21		23
5.0	699	549	549	528	14	66	66	62	24	34	34	38
7.5		641		616		56		52		44		48
10.0		733	733	708		49	49	44		51	51	56
19.0			1.072				30				70	
30.0			1.499				20*				80*	

Total energy content is in kcal/100 g tissue; protein and fat are in energy%
The Table reads as follows: plants with 5 g% fat contain 699 kcal/100 g, of which 14 en% is from protein and 24 en% from fat; the remainder (i.e. 62 en%; not shown) is from carbohydrates. The models are
composed of plant fat percentages varying from 2.5-5.0 g%; those of meat varied from 5.0-30.0 g% and of fish from 2.5-10 g%. 'Non selective' implies the following weight percentages: skeletal muscle 90.2 g%
containing 3.0 g% fat; brain 1.0 g% with 9.1 g% fat; bone marrow 3.0 g% with 51.0 g% fat; liver 3.8 g% with 6.7 g% fat and adipose tissue 2.0 g% with 84.2 g% fat. The cumulative fat content of this combination
amounts to 5.0 g%. 'Selective' implies some combination of muscle/marrow/brain. *, extrapolated (see text).

day hunter-gatherers (RSK and MFL; personal observations). In support of optimal foraging, it was recently shown that after fasting the human brain responded more actively to pictures of high-calorie foods compared to low-calorie foods.[30]

Plant composition

The macronutrient composition and caloric value of plant foods (Table 2) were derived from Eaton et al.[26] (fat 19 en%; protein 13 en% and carbohydrate 68 en%) and Cordain et al.[27] (fat 24 en%; protein 14 en% and carbohydrate 62 en%). They assumed a range from 469 kJ/100 g at 2.1 g% plant fat[26] to 699 kJ/100 g at 5 g% plant fat.[27,49] For Model 1 we adopted a 2.5 g% plant fat figure, while for Models 2-4, we varied the plant fat contents from 2.5-5 g%. Cordain[27] used a mean plant fat content of 5 g%, as derived from 829 wild plant foods consumed by Australian Aboriginals.[49] The fatty acid compositions of plant foods (Table 1) were derived from Guil et al.,[50] as also used by Eaton et al.,[26] with additional data for African vegetables such as terrestrial leaves, seeds, roots, tubers, nuts and fruits.[51-55] Edible seaweed, sea grasses and algae,[56-59] like insects,[60,61] were not included, but are shown for comparison in Supplemental Table 1. Marine, terrestrial plants and insects have comparable FA contents, except for the high LCP and lower precursor content in marine plants.

Justification for using the range of plant fat percentages in our models may come from examples of currently consumed plant foods in East-Africa. Tubers eaten by the Hadzabe in Tanzania contain up to 5 g% fat.[52] Although indigenous to North-America, Congolese tribes consume avocados with 19 g% fat.[55] Coconuts contain 46 g% fat and could have been available to our ancestors,[35] while palm nuts and peanuts contain 46 and 49 g% fat, respectively.[55] Nigerian wild plant seeds contain up to 59 g% fat,[53] nuts eaten by Australian Aborigines contain on average 29 g% fat[49] and the !Kung Bushmen consume fat-rich mongongonuts (57 g% fat) as staple food when available.[51] The preference of nuts or fatty fruits above low-fat leaves is consistent with optimal foraging.[29,30]

Meat composition

Non-selective meat consumption

Animal foods were subdivided into muscle, brain, bone marrow, liver and adipose tissue/separable fat. About 50% of the total body weight of game animals is edible.[62] For the calculation of the energy density and the energy contributions of protein and fat from an average edible portion of non-selectively consumed meat we assumed the following weight distribution and fat contents: skeletal muscle 90.2 g% containing 2.9 g% fat; brain 1.0 g% with 9.1 g% fat; liver 3.8 g% with 6.7 g% fat; bone marrow 3.0 g% with 51.0 g% fat and adipose tissue 2.0 g% with 84.2 g% fat.[26,45,47,55,63-70] The cumulative fat percentage of a non-selectively eaten portion of meat amounts to 4.89 g/100 g,[26] which for practical purposes was rounded to 5.0 g% fat, with a corresponding energy density of 549 kJ/100 g (see Table 2). For the various fat contents of non-selectively consumed meat (i.e. 2.5-10 g%) we calculated the energy densities and the energy contributions of protein and fat. For meat, the relations between energy density and fat content, and between energy from protein and fat

content, are linear, as previously noted by Cordain et al.[27] The employed relationships are:

$$\text{Energy density for meat (in kJ/g)} = 3.616 + 0.371 * \text{body fat (\% by wt)} \quad (1)$$

$$\text{Energy contribution for protein (in kJ/g)} = 96.79 - (7.92 * \text{body fat (\% by wt)}) + (0.403 * (\text{body fat (\% by wt)})^2) - (0.0090 * (\text{body fat (\% by wt)})^3)^{27} \quad (2)$$

The fat contents of meat (in en%) were calculated by taking 100 en% minus the protein en%. The final outcomes are in Table 2**.**

Selective meat consumption

The energy density and the contributions of protein and fat from an average portion of selectively consumed meat were calculated at 3 fat contents (i.e. 10.0; 19.0 and 30.0 g%). These fat percentages were derived from 3 different combinations of muscle, marrow and brain, i.e. 50/40/10, 20/64/16 and 0/80/20 en%/en%/en%, respectively. Justification for these combinations comes from the observation (see Results section) that protein intakes exceed the stated protein constraint (see below) from about 50 en% muscle consumption. Since brain and bone marrow have similar total weights (**Table 3**),[45-47] but bone marrow has about 4 times higher energy density (Table1), we kept the bone marrow/brain constant at a ratio of 4 en%/en%.

The calculations of the fat content (g%), energy from all macronutrients (kJ/100 g), and protein and fat contributions (in en%) may be illustrated as follows. A 20/64/16 en%/en%/en% ratio from muscle, bone marrow and brain, respectively, implies that for each 4,188 kJ selectively eaten meat of this composition 838, 2,680 and 670 kJ are derived from muscle, bone marrow and brain, respectively. Using the energy densities of these organs from Table 1, these energy quantities translate into 838/452 = 185 g muscle; 2,680/2,044 = 131 g bone marrow; and 670/528 = 127 g brain. These figures add to a total of 443 g selectively eaten meat, of which 41.7 g% is derived from muscle, 29.6 g% from marrow and 28.7 g% from brain. The total fat content of this 443 g portion was calculated by using the fat contents of the individual organs from Table 1, yielding 19 g% fat (see Table 2). The energy contribution from all macronutrients and the contribution from protein were subsequently calculated by using equations 1 and 2, respectively (see above), yielding 1,072 kJ/100 g selectively consumed meat and 30 en% from protein. The fat content (in en%) was calculated by taking 100 en% minus the protein en%. The fat content (g%), energy from all macronutrients (kJ/100 g), and protein and fat contributions (in en%) for the 50/40/10 and 0/80/20 en%/en%/en% compositions were calculated in a similar manner. These calculations needed extrapolation for the 0/80/20 composition, since the original data of Cordain's[27] equation 2 did not consider fat percentages above 25 g%. The final outcomes of the calculations for each of the organ combinations for selectively consumed meat are presented in Table 2.

Justification for the use of variable fat percentages in our models comes from the available data

Table 3. Average weight of whole animal. edible portion of whole animal and average weight of brain and marrow for African savannah animals

Animal	*Weight* (kg)	*Flesh* (% of wt))	*Brain* (g)	*Marrow* (g)
Thompson's Gazelle. adult	16.3	48		60
Thompson's, juvenile	12.5	47		41
Impala, adult	55.4	54	102	154
Grant's Gazelle, adult	57.3	53		202
Grant's, juvenile	12	53		34
Warthog, adult	61.4	55		91
Topi, adult	114	54		353
Hartebeest, adult	130	58	140	293
Wildebeest, adult	165	44	238	365
Zebra, adult	273		341	119

Data adapted from Blumenschine & Madrigal[45] and Lupo[46]

from East-African animal and hunter-gatherer studies. The nutrient composition of the various tissue compartments in animals is variable. For instance, the fat content of skeletal muscle from game animals is much lower than that of livestock[47,64,71] and ranged from 2.0 g% in the Ugandan Eland[68] to 4.6 g% in monkey bush meat from Zaire[55] in our database, but can be as high as 25 g% in domestic cattle.[64] The fat content of bone marrow is strongly dependent upon season and the animal's age and physical condition.[45,47] Also the size of the adipose tissue mass of game animals is dependent on season, condition and age.[45-47] In contrast to the above organs, the fat contents of liver and brain are rather constant.[47] In view of this variance and the optimal foraging theory[29,30] we varied the average fat content of the consumed whole carcass edible meat from 5.0-10.0 en% and for (very) selective organ consumption in Models 1 and 3 to a maximum of 30.0 en% to calculate the caloric value and the macronutrient and fatty acid compositions of the *possible* diets. It is important to realize that the consumption of 'meat' containing 30 g% fat does not refer to consumption of lean muscle meat only, since the maximum lean meat fat% of some East African mammal species was 13.0 g% in a female hippopotamus in Ledger's classical study.[62] We rather point at the selective consumption of fatty organs as the brain and bone marrow or liver and adipose tissue. Secondly, selective hunting would be part of optimal foraging strategies,[29] meaning that hunter-gatherers would spend more effort in hunting fat than in hunting lean animals. With reference to the employment of a wide range of dietary fat, we emphasize that the current study is rather designed to show the range of possible dietary intakes from a constantly changing environment in the past, than to point at one specific dietary composition.

Fish composition

At various fish body fat contents (i.e. 2.5; 5.0; 7.5 and 10.0 g%) we calculated the energy density and the contributions of protein and fat (in en%). We found that the energy densities for fish

from the USDA food database[33] correlate excellently with the corresponding body fat contents (**Figure 1**). Using the linear relationship: energy density (kJ/g) = 0.366 * body fat (in g%) + 3.425 (R^2 = 0.94) we subsequently calculated the energy densities at the various fish body fat contents. The corresponding energy contributions for protein were calculated from the following equation:

$$\text{Protein (en\%)} = 97.67 - (9.45 * \text{body fat (g\%)}) + (0.535 * (\text{body fat (g\%)})^2) - (0.0127 * (\text{body fat (g\%)})^3)^{27} \quad (3)$$

The fish fat contents (in en%) were calculated by taking 100 en% minus the protein en%. The final outcomes are in Table 2**.**

Justification for the employed fat percentages from fish may come from the following data. The fat content of most pelagic fish is around 2.5 g%, but African catfish have fat contents above 10 g%.[72] In most studies, the fat content is derived from analyses of the fillet, while Pauletto et al.[72] specifically examined a portion including the fat-rich skin (37-44 g% of total fat).[73] In addition to the consumption of skin, the first part of a fish to be eaten by Africans is the head, which contains 10-17 g% of total fat.[73] Another 3.5-6 g% of fat may come from the consumption of the backbone.[73] Although large fish were reportedly caught with bare hands back to 2 million years ago,[9] especially in smaller fish the skin would have been a substantial part of the total edible portion. For instance we estimate that 2-20 g% fat would be derived from the whole consumption of a 10 cm fish with a diameter of 3 cm and a 2 mm skin. Taking these assumptions into account for the approximation of the average fat content of the fish consumed by early humans, we varied the average fish fat content in our models from 2.5-10.0 g%. Since 'you are what you eat' also applies to fish, we only

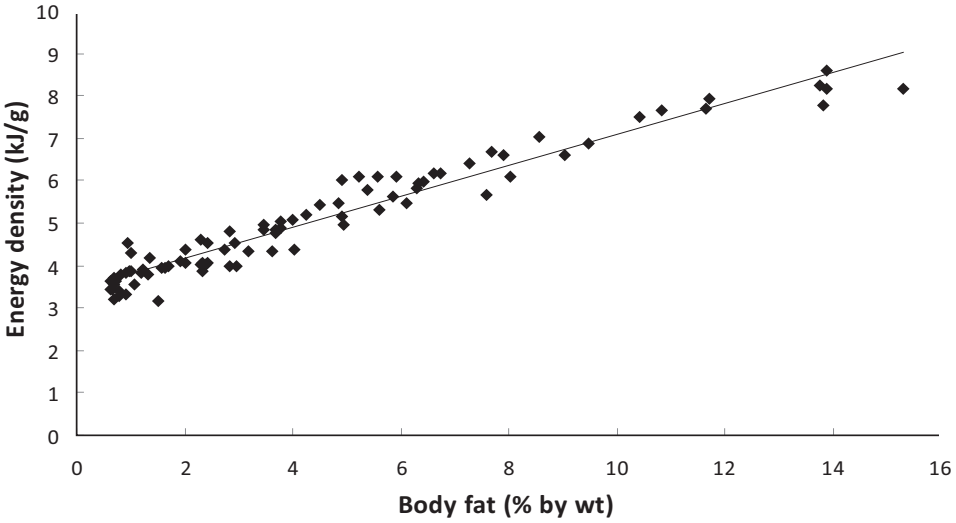

Figure 1. Relation between body fat (in g%) and energy density (in kJ/g) for fish. All the data on freshwater fish are derived from the USDA internet database (n=87), accessed on 15 August 2008.[33] y = 0.0366x + 3.425, R^2 = 0.94

used fatty acid data on East-African fish species. The differential fatty acid status of worldwide and East-African fish species[10,34,35,72,73] are presented in supplemental Table 1. Data for the FA composition of some other typical marine animals, such as crustaceans, cephalopods and certain marine reptiles, birds, mammals, including their eggs, livers and adipose tissue are presented in Supplemental Table 1, but are not included in the models. The FA compositions are quite similar to that of the employed African fish species.

Calculation example

The example assumes a 12,500 kJ/day diet that is composed of 70 en% plant (containing 2.5 g% fat) and 30 en% animal food. The latter is composed 15 en% fish with 2.5 g% fat and 15 en% meat. The meat was either non-selectively consumed meat with 5 g% fat, or selectively eaten meat with 19 g% fat.

Macronutrient composition

The plant protein contribution in this example would be 12,500 kJ/day * 70 en% * 13 en% (Table 2) = 1.143 kJ/day. Similarly, the protein contribution from meat would be 12,500 * 15% * 66% (Table 2) = 1,244 kJ/day, while the protein contribution from fish is 12,500 * 15% * 77% (Table 2) = 1,453 kJ/day. Taken together, the total protein intake from this 12,500 kJ/day diet would be 1,143 + 1,244 + 1,453 = 3,840 kJ/day, which equals 30.6 en% of total energy intake. The corresponding fat and carbohydrate intakes were 2,747 kJ/day (21.9 en%) and 5,980 kJ/day (47.5 en%), respectively.

The macronutrient contribution for 19% fat in selectively consumed meat is calculated in a similar manner. The plant protein contribution would again be 1,143 kJ/day (see above). The protein contribution from meat would be 12,500 * 15% * 30% (Table 2) = 565 kJ/day, while the contribution from fish protein would again be 1,453 kJ/day (see above). Taken together, the total protein intake from this 12,500 kJ/day diet would be 1,143 + 565 + 1,453 = 3,161 kJ/day, which equals 25.2 en% of total energy intake. The corresponding fat and carbohydrate intakes were 3,425 kJ/day (27.3 en%) and 5,980 kJ/day (47.5 en%) respectively.

Fatty acid composition: arachidonic acid as an example

Table 4 shows the outcome of the arachidonic acid (AA) content per 100 g of consumed meat, as calculated by assuming non-selective and selective meat consumption, respectively. Data for all other fatty acids in Table 1 were also calculated (not shown). The employed AA data in plants and fish are presented in Table 1.

A 12,500 kJ/day diet with 70 en% from plants corresponds with 8,794 kJ/day from plants. The plant energy density at 2.5 g% fat is 469 kJ/100 g (Table 2), which translates the 8,794 kJ/day into an intake of 8,794/4.69 = 1,875 g plants/day. The plants were assumed to contain 2.5 g% fat with an AA content of 0.64 g/100 g fat (Table 1), which implies a total daily AA intake of 1,875 * 2.5/100 * 0.64/100 = 0.30 g from plants. Similarly, 15 en% from fish with 2.5 g% fat translates into 12,500

Table 4. Arachidonic acid AA) content of 100 g consumed meat assuming non-selective (whole carcass) consumption or selective organ consumption.

Tissue	*Organ[a] (g)*	*Fat[b] (g%)*	*AA[c] (g%)*	*AA[d] (mg)*
Non-selective consumption[e]				
Muscle	90.2	2.9	6.40	167
Brain	1.0	9.1	5.74	5
Liver	3.8	6.7	9.40	24
Bone marrow	3.0	51.0	0.16	2
Adipose tissue	2.0	84.2	0.21	3
Total	100.0			203
Selective consumption[f]				
Muscle	41.7	2.9	6.40	80
Brain	28.7	9.1	5.74	150
Liver	0.0	6.7	9.40	0
Bone marrow	29.6	51.0	0.16	24
Adipose tissue	0.0	84.2	0.21	0
Total	100.0			254

[a], Organ, contribution (in g) of the indicated organ to the consumption of 100 g meat at different foraging strategies
[b], Fat, gram fat per 100 g of indicated organ
[c], AA: gram AA per 100 g fat in indicated organ
[d], calculated
[e], data from Eaton *et al.*[26] (organ contribution) and the literature (fat g% and AA g%; see suppl. Table 1)
[f], calculated (organ contribution) and data from the literature (fat g% and AA g%; supplemental Table 1)

* 15/100 * 100/436 (Table 2) * 2.5/100 * 8.45/100 = 0.91 g AA per day. The daily amount of meat from non-selective consumption at 15 en% would be: 12,500 * 15/100 * 100/549 (Table 2) = 344 g meat. With an average content of 203 mg AA per 100 g non-selectively eaten meat (Table 4), this figure adds up to 344 * 0.203/100 = 0.70 g AA per day. Assuming selective consumption of meat the daily intake of AA would become: 12,500 * 15/100 * 100/1,072 (Table 2) * 0.254/100 (Table 4) = 0.45 g. Taken together, the intake of AA in this example from plants, fish and non-selectively consumed meat would be 0.30 + 0.91 + 0.70 = 1.91 g, while for selective meat consumption the intake would be 0.30 + 0.92 + 0.45 = 1.67 g AA/day. Additional calculation of the daily intakes of all other fatty acids and subsequent normalization to g/100 g fatty acids gave rise to the dietary fatty acid composition (detailed data not shown).

Constraints

Since not all dietary combinations are compatible with good health, we introduced two pathophysiological constraints. First the contribution of protein is not to exceed 35 en%, since this may cause 'rabbit starvation', probably by exceeding the maximum capacity of the liver to convert the excess nitrogen into urea.[74] Some studies even suggest that in adult male hunter-gatherers the protein intake may be closer to 40-50 en%.[75] The constraint to restrict the average protein intake at 35 en% seems, however, justified by hunter-gatherer observations.[76] Second, LA intakes were to be

above 1.0 en% to prevent LA deficiency, especially in children. This constraint is derived from the original data of Burr and Burr;[77] as revisited by Cuthbertson,[78] who even stated that 'the minimum requirements for LA are in fact less than 0.5% of calories;' and set at a minimum requirement of at least 1.0% of calories as LA to prevent biochemical LA-deficiency. In animals, the minimum requirement for LA could also be met by 18:3ω6 and AA,[79] which would actually imply that the currently employed constraint for LA would be superfluous for any diet containing substantial amounts of LCPω6 (see also discussion). In addition to these two constraints we tested whether the reconstructed diets provide the daily intake of 450-500 mg EPA+DHA per day to lower CAD risk, as recommended by e.g. the UK Scientific Advisory Committee on Nutrition, the WHO and ISSFAL.[80]

RESULTS

Means and ranges of the models

The extremes for Models 1, 2 and 3, i.e. the 70/30 and 30/70 en%/en% plant/animal subsistence ratios, are depicted in **Figures 2 and 3**, respectively. For Model 1 a total of 105 different diets were evaluated. Of these 71 (68%) met the protein constraint, where after all models met the LA constraint and the EPA+DHA recommendation. For Model 2 we evaluated a total of 1,320 different diets; 674 (51%) met the protein constraint and thereafter all models met the LA constraints and the EPA+DHA recommendation. Model 3 considered a total of 165 different diets; 115 (70%) met the protein constraint, where after again all models met the LA constraint and the EPA+DHA recommendation. Model 4 is an extreme of Model 2 (or 3) and describes plant and fish intakes only. Since the model is two, instead of multidimensional, it is not depicted separately. The model evaluated 40 options, for which 22 (55%) met the protein constraint where after all models met the LA constraint and the EPA+DHA recommendation.

Model 1 in Figure 2 represents data for a 70/30 en%/en% plant/animal subsistence ratio, assuming the following fat contents: plants 2.1 g%, muscle 3.0 g%, bone marrow 51 g% and brain 9.1 g%. The contribution of muscle/marrow/brain to the consumed meat (in en%) was varied from 98/1/1 to 0/80/20 en%/en%/en%. The short hand notations of these extremes (see x-axis labels) would be 70/98/1/1 to 70/0/80/20, in which the first figure represents the en% from plants and the last three figures the en% contributions from muscle/marrow/brain in the remaining 30 en% animal food (i.e. only meat in Model 1), all at the fixed fat contents (in g/100 g material). The data in this example show that replacing muscle for bone marrow and brain (i.e. x-axis from left to right) causes a decrease in the contributions of LA and protein (both in en%), and increases in the ALA/LA and (EPA+DHA)/AA ratios (both in g/g). All investigated meat compositions within the depicted 70/30 en%/en% plant/animal example of Model 1 complied with the <35 en% protein, >1.0 en% LA and met the >450 mg EPA+DHA recommendation.

Similarly, **Figure 3** shows data for Model 1 at the 30/70 en%/en% plant/animal subsistence ratio. The same plant, muscle/marrow/brain fat contents and the same animal compositions were used. The protein constraint was met from a ratio of 30/50/40/10. All animal compositions complied with

the energy constraints for LA and the 450 mg EPA+DHA recommendation.

For Model 2, examples are shown for 5 g% fat in plants, 5.0 g% fat in fish and 7.5 g% fat in whole carcass meat (Model 2, Figures 2 and 3, left panels) and for 5 g% fat in plants, 7.5 g% fat in fish and 5.0 g% fat in whole carcass meat (Model 2, Figures 2 and 3, right panels). The meat/fish compositions were varied from 100/0 to 0/100 en%/en% of total animal food. In the left panel for Model 2 in Figure 2, compliance with the protein and LA constraints and the EPA+DHA recommendation was reached for all dietary compositions.

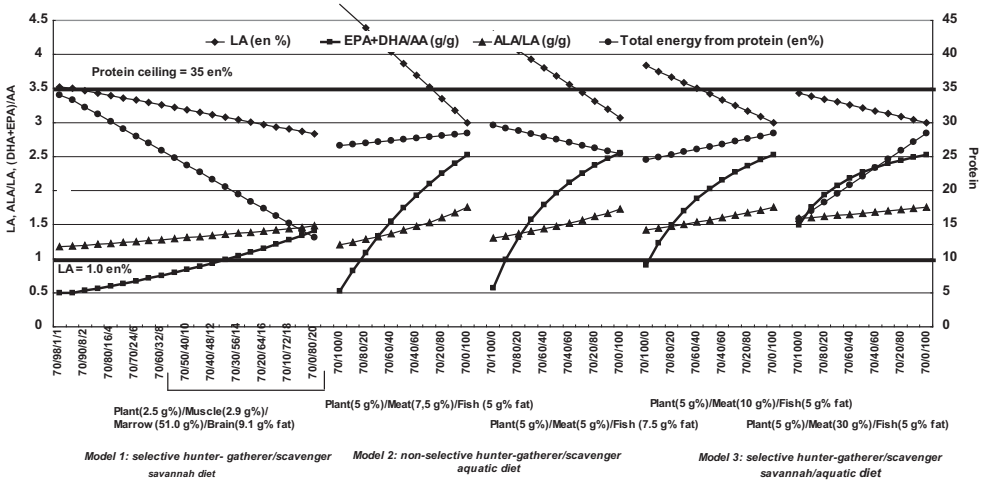

Figure 2. The courses of the protein (total energy from protein, -●-, en%) and linoleic acid (LA) intakes (-◆-, in en%) and the α-linolenic acid (ALA)/LA (-▲-) and EPA + DHA)/arachidonic acid (AA) ratios (-■-, in g/g) with changing composition of animal food at a 70/30 en%/en% plant/animal subsistence ratio. Animal food was composed of organ meat (skeletal muscle, brain, bone marrow, liver and adipose tissue) and fish. Meat consumption was either selective (Models 1 and 3) or non-selective (Model 2). The shorthand notation on the X-axes indicates plant/muscle/marrow/brain (Model 1) and plant/meat/fish (Models 2 and 3). Note the differences of the left and right scales of the Y-axes. The range of dietary combinations within the box in Model 1 was used for the construction of Model 3 (see text). Horizontal lines depict the employed protein (<35 en%) and LA (>1.0 en%) constraints. The data for Model 1 (a selective hunter-gatherer/scavenger savannah diet) were calculated by assuming the selective consumption of plant, muscle, bone marrow and brain with the fat contents of 2.5, 2.9, 51.0 and 9.1 g%, respectively. Their intakes were varied between 98 and 0 en% (skeletal muscle), 1 and 80 en% (bone marrow) and 1 and 20 en% (brain) of the total meat intake. The data for Model 2 (a non-selective hunter-gatherer/scavenger aquatic diet) were calculated by assuming non-selective consumption of edible meat with the following weight distribution and fat contents: skeletal muscle 90.2 g% containing 2.9 g% fat; brain 1.0 g% with 9.1 g% fat; bone marrow 3.0 g% with 51.0 g% fat; liver 3.8 g% with 6.7 g% and adipose tissue 2.0 g% with 84.2 g% fat.[25,45,47,55,62-68,70] The fat percentages were 5 g% for plants 5.0 g% for fish and 7.5 g% for whole carcass meat (left panel of Model 2), and 5 g% for plants, 7.5 g% for fish and 5.0 g% for whole carcass meat (right panel of Model 2). The data for Model 3 (a selective hunter-gatherer/scavenger savannah/aquatic diet) were calculated by assuming the selective consumption of muscle, bone marrow and brain with the fat contents of 2.9, 51.0 and 9.1 g%, respectively. The meat and fish intakes were varied from 100 to 0 and 0 to 100 en% of total animal consumption, respectively. The intakes from muscle, bone marrow and brain were varied (from left to right panel of Model 3) from 0 to 50 en% (muscle), 40 to 80 en% (bone marrow) and 10 to 20 en% (brain) of total meat intake. The fat contents of fish and plants were both set at 5 g%, while the average fat contents of the combined muscle/marrow/brain in meat varied from 10 (left panel of Model 3) to 30 g% (right panel of Model 3).

Finally, for Model 3, examples are given again for the 70/30 en%/en% (Figure 2) and 30/70 en%/en% (Figure 3) plant/animal subsistence ratios, but here at 50/40/10 (left panels) and 0/80/20 (right panels) for the muscle/marrow/brain ratios. The fat contents were: 5 g% for plants, 10 g% for meat and 5 g% for fish (left panels), and 5 g% for plants, 30 g% for meat and 5 g% for fish (right panels). Justifications for these fat percentages came from the application of the protein constraints to Model 1 in Figure 3, which fixed the muscle/marrow/brain ratios in meat between 50/40/10 and 0/80/20, and consequently also fixed the fat percentages.

Table 4 presents the medians (ranges) from all investigated models together with the original data from the savannah diet of Eaton et al.[26] for a 65/35 en/en% plant/animal

subsistence ratio and from Cordain et al.[27] for a 45/55 en/en% plant/animal subsistence ratio. All presented data from Model 1 complied with the protein and LA constraint and the EPA+DHA recommendation. All presented data of Models 1-4 (**Table 5**) complied with the protein and LA constraints and, after application of these constraints, all models also met the EPA+DHA recommendation.

Model outcomes

Model 1, selective hunter-gatherer/scavenger savannah diet

All the models met the EPA+DHA recommendation. It was found that with the exception of the 70/30 en%/en% plant/animal subsistence ratio, all investigated dietary options were limited by the 35 en% protein ceiling. This ceiling was reached when more than 45 en% of animal food was consumed as muscle meat. LA, AA and EPA+DHA intakes increased with decreasing plant intakes, whereas ALA intakes decreased. At low plant intakes, only those options with high intakes of bone

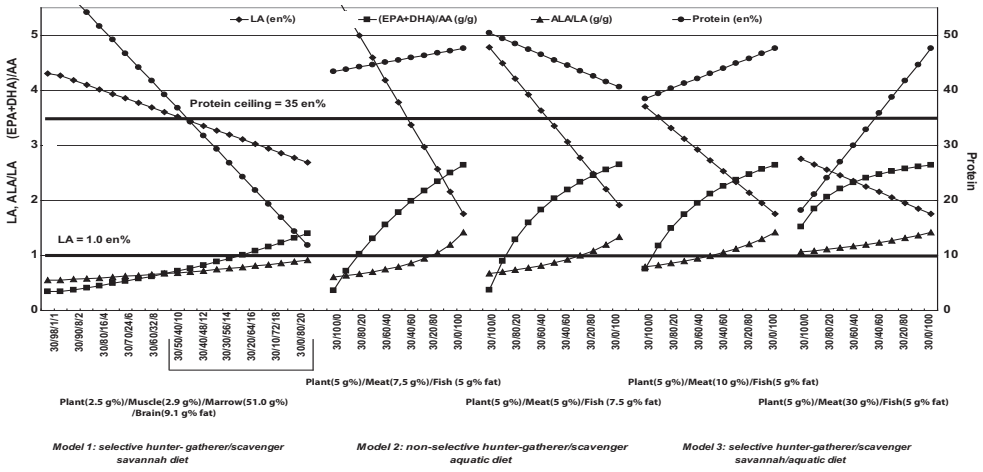

Figure 3. The courses of the protein (total energy from protein, -●-, en%) and linoleic acid (LA) intakes (-◆-, in en%) and the α-linolenic acid (ALA)/LA (-▲-) and EPA + DHA)/arachidonic acid (AA) ratios (-■-, in g/g) with changing composition of animal food at a 30/70 en%/en% plant/animal subsistence ratio. For legend: see Figure 2.

marrow and brain, relative to muscle, fulfilled the <35 en% protein constraint. At 70 en% intake from plants, 0 en% from muscle, 80 en% from bone marrow and 20 en% from brain, the intakes of bone marrow and brain would be 141 and 148 g/day, respectively. At he lowest intake of plants (i.e. 30 en%) and at contributions of muscle, bone marrow and brain ranging from 0-50, 40-80 and 10-20 en% respectively, the intakes on a weight basis for muscle, bone marrow and brain ranged from 0-867, 189-344 and 181-328 g/day, respectively. LA intakes ranged from 2.83-3.52 en% in a 70 en% plant food diet and from 2.69-4.30 en% in a 30 en% plant food diet. With protein and LA complying with their recommendations the intakes of EPA+DHA ranged from 0.88 -1.63 g/day at 70 en% plant food, and from 2.20-2.98 g/day at 30 en% plant food, while the (EPA+DHA)/AA ratios ranged from 0.41-1.41 and 0.77-1.40 g/g, respectively.

Model 2, non-selective hunter-gatherer/scavenger savannah/aquatic diet

In this model, several plant/(meat/fish) ratios that were investigated at different fat percentages fulfilled the two constraints. All options that met these constraints also met the EPA+DHA recommendation. Also here, the options became restricted by the protein ceiling, when the intake of animal food increased. Reaching the protein ceiling proved dependent on the fat contents of the consumed meat and fish, as can be concluded from Figures 2 and 3. Not unexpectedly, these indicate that the lower the fat content of the animal foods (i.e. the leaner the meat), the sooner the protein ceiling is reached. Since LA is relatively abundant in plants, muscle and liver, but not fish (Table 1), the constraint for LA (>1.0 en%) could not be reached if 70 en% was consumed as animal food that is composed of high fat meat (e.g. 100 en% meat with ≥ 7.5 g% fat) in combination with low fat plants (e.g. 2.5 g% fat) (Figure 2, left panel for Model 2). Models 2 in Figures 2 and 3 show that the slope of the (EPA+DHA)/AA curve steepens with increasing fat content in fish and decreasing fat content in meat. This steepening of the (EPA+DHA)/AA curve takes place at an increase of both the EPA+DHA and AA intakes, indicating that the EPA+DHA content of the various dietary options, and not the AA content, is the most variable factor determining the (EPA+DHA)/AA ratio in Model 2.

Model 3, *selective hunter-gatherer/scavenger savannah/aquatic diet*

Model 3 combines the favorable foraging strategies for hunting, gathering and scavenging from Models 1 and 2. All dietary options that met the constraints for protein and LA also met the EPA+DHA recommendation (Table 5). The protein ceiling becomes limiting at the combination of low plant intake and the consumption of low fat meat, as can be derived from Figure 3 (left panel). This limitation became circumvented by the consumption of high fat meat (Figure 3, right panel), while it also becomes circumvented by the consumption of high fat plants and fish (data not shown).

Model 4, non-selective hunter-gatherer/scavenger aquatic diet

In this model, 55% of the investigated plant/fish ratios that were investigated at different fat percentages fulfilled the protein constraint. All options meeting this constraint also met the LA constraint and the EPA+DHA recommendation.

Table 5. Reconstructed Paleolithic diets at different foraging strategies*

	Meat based (non-selective‖)	Meat based (non-selective§)	Meat based (selective; Model 1)	Fish/meat based (non-selective; Model 2)	Fish/meat based (selective; Model 3)	Fish based (non-selective; Model 4)
Nutrient	Median	Median	Median(Range)	Median(Range)	Median(Range)	Median(Range)
Animal/plant subsistence ratio						
Plant/animal (en%/en%)	65/35†	45/55§	50/50(70-30/30-70)	50/50(70-30/30-70)	55/45(70-30/30-70)	57/43(70-30/30-70)
Meat/fish (en%/en%)	100/0	100/0	100/0	60/40(0-100/100-0)	58/52(0-100/100-0)	0/100
Muscle/marrow/brain (en%/en%/en%)	-	-	30/56/14(98/1/1-0/80/20)	-	20/64/16(50/40/10-0/80/20)	-
Plants (g/day)	1653‖	988	1607(804-1875)	1257(539-1875)	1078(539-1257)	1257(534-1875)
Meat (g/day)	890‖	1031	695(288-1213)	360(0-1028)	128(0-411)	0
Fish (g/day)	0	0	0	381(0-1243)	681(357-952)	867(533-1243)
Macronutrients						
Energy (kJ/d)	12,500†	12,500§	12,500	12,500	12,500	12,500
Protein (en%)	37‡	30§	25(8-35)	29(22-35)	27(16-35)	29(22-35)
from plants (en%)	9.2	5.4	8.4(4-10)	8.4(4-10)	7.0(4-10)	7.7(4-10)
from animal (en%)	29	25	16.9(0-32)	21(14-40)	19(6-29)	22(13-31)
Carbohydrate (en%)	41‡	34§	40(20-47)	40(19-48)	40(20-47)	39(19-48)
Fat (en%)	22‡	36§	39(21-72)	30(20-46)	34(25-62)	34(20-46)
Protein (g/d)	236	191	160(51-223)	185(140-223)	172(102-223)	185(140-223)
from plants (g/d)	60¶	-	54(26-64)	54(27-63)	45(27-63)	49(26-64)
from animal (g/d)	191¶	-	108(0-204)	134(92-253)	119(38-185)	140(83-198)
Carbohydrate (g/d)	294	243	287(144-337)	287(136-345)	287(144-337)	280(136-344)
Fat (g/d)	70	114	124(67-229)	95(64-146)	108(79-197)	108(64-146)
Essential fatty acids						
ALA (18:3ω3. g/d)	12.6‖	15.0	11.9(7.73-13.4)	13.5(6.57-18.5)	14.8(8.63-17.4)	12.6(6.57-17.0)
EPA (20:5ω3. g/d)	0.39‖	0.71	0.38(0.14-0.59)	1.74(0.56-6.61)	1.41(0.30-2.80)	3.45(1.41-6.61)
DPA (22:5ω3. g/d)	0.42‖	0.96	0.52(0.20-0.90)	1.53(0.66-4.71)	1.03(0.20-1.93)	2.36(0.89-4.71)

DHA (22:6ω3. g/d)	0.27‖	0.41	1.35(0.29-2.84)	4.30(0.32-21.7)	4.36(0.81-8.79)	10.8(3.93-21.7)
EPA+DHA (g/d)	0.66‖	1.12	1.70(0.87-2.98)	6.10(0.88-28.3)	5.83(1.38-11.6)	14.2(5.34-28.3)
LCPω3 (g/d)	1.61	2.01	2.26(1.53-3.52)	7.64(1.47-33.9)	6.89(1.76-13.8)	17.0(6.33-33.9)
ω3 (g/d)	17.5	20.3	16.6(12.2-18.5)	25.9(16.0-44.4)	25.2(14.3-31.9)	34.1(22.1-44.4)
LA (18:2ω6, g/d)	8.84‖	14.3	9.98(8.60-11.2)	11.3(5.53-19.8)	9.83(7.20-12.2)	7.46(5.53-9.96)
AA (20:4ω6, g/d)	1.81‖	2.41	1.81(1.15-2.77)	3.65(1.69-10.7)	2.84(1.15-4.61)	5.46(2.14-10.7)
LCPω6 (g/d)	2.23	2.81	2.54(2.03-3.99)	5.09(2.00-17.4)	4.51(1.91-7.64)	8.84(3.41-17.4)
ω6	14.8	17.9	13.4(11.7-15.6)	17.9(10.9-25.9)	15.2(12.9-16.2)	17.6(10.9-24.2)
LCP (g/d)	3.75	4.70	4.75(3.46-7.46)	12.5(3.38-51.3)	11.2(3.77-21.2)	25.8(9.74-51.3)
ALA/LA (g/g)	0.70	1.04	1.12(0.70-1.56)	1.25(0.61-1.79)	1.47(0.93-1.75)	1.64(1.19-1.79)
(EPA+DHA)/AA (g/g)	0.49	0.47	0.95(0.49-1.41)	1.82(0.36-2.66)	2.13(0.78-2.58)	2.60(2.45-2.66)
LCPω3/LCPω6 (g/g)	0.72	0.72	0.84(0.74-0.92)	1.56(0.67-1.96)	1.86(0.22-3.07)	1.92(1.83-1.96)
ω3/ω6 (g/g)	1.19	1.13	1.22(0.79-1.59)	1.50(0.66-2.05)	1.69(1.01-2.01)	1.94(1.82-2.05)
ALA (18:3ω3, en%)	4.0	4.7	3.7(2.4-4.2)	4.2(2.1-5.8)	4.7(2.7-5.5)	4.0(2.1-5.3)
LCPω3 (en%)	0.5	0.6	0.7(0.5-1.1)	2.4(0.5-11)	2.9(3.9-4.9)	2.9(3.9-4.9)
LA (18:2ω6, en%)	2.8	4.5	3.1(2.7-3.5)	3.6(1.7-6.2)	3.1(2.3-3.8)	2.3(1.7-3.1)
LCPω6 (en%)	0.7	0.9	0.8(0.6-1.3)	1.6(0.6-5.5)	1.4(0.6-2.4)	2.8(1.1-5.5)
Other fatty acids and cholesterol						
SAFA (g/d)	31.7	38.8	36.3(23.0-56.1)	38.1(21.8-59.1)	36.4(31.6-51.6)	38.0(21.8-53.9)
MUFA (g/d)	23.4	29.2	58.8(11.5-124)	23.6(9.90-50.1)	41.3(14.5-109)	17.9(9.90-26.5)
PUFA (g/d)	29.8	34.4	27.2(25.7-28.2)	40.2(28.9-66.6)	36.4(26.4-43.9)	48.3(29.5-66.6)
P/S ratio (g/g)	1.40‡	1.10	0.75(0.46-1.23)	1.07(0.68-1.37)	1.05(0.58-1.34)	1.30(1.23-1.37)
Cholesterol (mg/d)	480‡	830	3138(651-6910)	498(321-748)	914(430-3107)	523(321-748)
SAFA (en%)	10.0	12.2	11.4(7.2-18)	12.0(6.9-19)	11.5(9.9-16)	12.0(6.8-17)
MUFA (en%)	7.4	9.2	18.5(3.6-39)	7.4(3.1-16)	13.0(4.6-34)	5.6(3.1-8.3)
PUFA (en%)	9.4	10.8	8.6(8.1-8.9)	12.6(9.1-21)	11.5(8.3-14)	15.2(9.3-21)
ω3 (en%)	5.5	6.4	5.2(3.9-5.8)	8.1(5.0-14)	7.9(4.5-10)	10.7(7.0-14)
ω6 (en%)	4.7	5.6	4.2(3.7-4.9)	5.6(3.4-8.1)	4.8(4.1-5.1)	5.6(3.4-7.6)

Legend table 5
ALA, α-linolenic acid; DPA, docosapentaenoic acid; LCP, long-chain PUFA; LA, linoleic acid; AA, arachidonic acid; P/S, polyunsaturated/saturated.
* Values in the models of Eaton *et al.*[26] and Cordain *et al.*[27] without reference were calculated by using the present models. Protein, carbohydrate and fat contents were converted into kJ/g using conversion factors of 19.68; 17.50 and 39.53 kJ/g, respectively. Some of the calculated data on LCP, LCPω3 and LCPω6 from the literature may suffer from underestimation, since authors seldomly give information on the complete fatty acid profile used to calculate these sums. Also for some fatty acid contents, more data were available in the literature than for others. As a consequence, for example, the sum of EPA+DPA+DHA (all regularly given) might be higher than the sum of LCPω3, which is seldomly given.
† Adapted from Eaton *et al.*[2]
‡ Adapted from Eaton *et al.*[3]
‖ Adapted from Eaton *et al.*[26]
§ Adapted from Cordain *et al.*[27]
¶ Adapted from Frassetto *et al.*[122]

DISCUSSION

We estimated the median and ranges of the dietary macronutrient and fatty acid compositions for multiple foraging strategies ascribed to Paleolithic hunter-gatherer/scavengers living in the savannah, the water-land ecosystem and combinations of these. Most importantly we found that the macronutrient composition averaged 25-29 (range 8-35) en% from protein, 39-40 (range 19-48) en% from carbohydrate and 30-39 en% (range: 20-72) from fat. These outcomes indicate moderate-to-high protein and fat intakes, with moderate carbohydrate intakes. Compared to current Western intakes and recommendations, the fatty acid composition was high in SAFA (range of medians 11.4-12.0; total range 6.8-19 en%), and moderate-to-high in MUFA (5.6-18.5; 3.1-39 en%) and PUFA (8.6-15.2; 8.1-21 en%). The PUFA were high in ALA (3.7-4.7; 2.1-5.8 en%), low in LA (2.3-3.6; 1.7-6.2 en%), and high in LCP (4.75-25.8; 3.38-51.3 g/day); both LCPω3 (2.26-17.0; 1.47-33.9 g/day) and LCPω6 (2.54-8.84; 1.91-17.4 g/day). Consequently, the ALA/LA ratio (1.12-1.64; 0.61-1.79 g/g) was remarkably higher compared to the present ALA/LA ratio (ALA/LA=0.09; .[81] The LCPω3/LCPω6 (0.84-1.92; 0.22-3.07 g/g) ratio was comparable to the current (0.85;[81]), but the absolute intakes of both LCPω3 and LCPω6 were remarkably higher. A comparison with other employed diets,[18,82,83] some current worldwide intakes[81,84-87] and recommendations,[88-90] and a qualification of the macronutrient,[91] fatty acid and cholesterol composition is given in **Table 6**.

Our ecological niche

From 1.9 million to 200,000 years ago hominins have tripled their brain mass relative to body mass, as usually expressed in terms of the encephalization quotient (EQ). The predominantly vegetarian *Australopithecines* were estimated to have an EQ of 1.23-1.92, while the *Homo* genus has an EQ of 1.41-4.26.[10] The *Ardipithecus ramidus* may have persisted in a more closed wooded habitat,[92] but the *Australopithecines* are assumed to have left the forest to enter the open[93] where they were able to introduce more energy dense animal food into their diets at the expense of energy poor plants.[94] Improvement of dietary density and quality may have enabled an increase in brain size, while it also provided the higher energy needs for the expanding, metabolically expensive, brain. It has been

Table 6: The macronutrient, fatty acid and cholesterol composition of different currently employed diets, our average current diet, some stated recommendations and a qualification of the macronutrient, fatty acid and cholesterol composition.

Macronutrient	Unit	Paleo	Atkins[a]	South Beach[a]	Zone[a]	Mediter-renean[a]	Learn[b]	DASH[a]	Ornish[a]	Current intake	Recom-mendation	Keto-genic[c]	Low	Moderate	High
Protein	en%	25-29	29	26	35	16	19	20	18	15-16[d]	10-35		<10[e]	10-25	>25
Carbohydrate	en%	39-40	9	33	37	38	48	53	75	49-52[d]	40-65	<10	10-25[e]	26-45[e]	>45[e]
Fat	en%	30-39	62	40	27	46	31	28	7	33[d]	20-35		<20[e]	20-35[e]	>35[e]
Fatty acids															
SAFA	en%	11.4-12.0	20	11	7	11		7	7	11-12[f]	<7[g,h]		<5	5-10	>10
MUFA	en%	5.6-18.5	23	18	13	28		12	2	13[f]			<5	5-15	>15
PUFA	en%	8.6-15.2	12	8	6	7		7	2	5-7[f,i]	2x/wk oily fish[g,h]		<5	5-10	>10
ALA	en%	3.7-4.7				0.8[j]				0.6[f]	0.6-1.2[e]		<1	1-3	>3
EPA+DHA	g/day	1.70-14.2								0.11[f]	0.5[h]		<0.25	0.25-0.50	>0.50
LA	en%	2.3-3.6				3.6[j]				6-7[f]	5-10[e]		<5	5-10	>10
AA	g/day	1.81-5.46								0.09[k]			<0.25	0.25-0.50	>0.50
Trans-fat	en%	0	<1	<1	<1	<1		<1	<1	2-3[l]	<1[g]				
Cholesterol	mg/day	498-3138	731	221	208	337		150	6	201[k]	<200-300[g,h]		<300	300-600	>600

[a], de Souza *et al.*[83]; [b], Gardner *et al.*[82]; [c], Feinman *et al.*[91]; [d], www.cdc.gov[84]; [e], www.iom.edu[88]; [f], Ramsden *et al.*[85]; [g], www.heart.org[89]; [h], www.diabetes.org[90]; [i],Smit *et al.*[86]; [j], de Lorgeril *et al.*[18]; [k], Ervin *et al.*[81]; [l], Mozaffarian *et al.*[87].Abbreviations: en%, energy%; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; wk, week; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid.

hypothesized that brain growth was preceded by the development of a sizeable adipose tissue compartment[95] to ensure continuous availability of energy, which is also known as 'the survival of the fattest'.[96] Other physical adjustments might have been necessary, since the adult primate brain usually consumes 8-9 % of the total resting metabolic rate, while this amounts to 20-25 % in anatomically modern humans.[97] One of these adjustments is the loss of muscle.[97] Additional energy reallocation might have come from adjustment of the gastrointestinal tract. The size of our current gastrointestinal tract comprises only 60% of expected for a similarly-sized primate, probably because of its devotion to easily-digested energy dense foods rather than a fiber-rich, bulky and consequently energy-poor vegetarian diet that requires a large colon.[98] The trade-off with the energetically expensive gastrointestinal tract is also known as the 'expensive tissue hypothesis' of Aiello and Wheeler.[99]

Brain expansion not only necessitates energy for its growth and maintenance, but also the availability of building blocks such as AA and DHA, and many other factors, collectively referred to as 'brain-selective nutrients'.[10,15] The question is what ecological niche would have supported the growth of our brain and especially the increment of DHA within a species characterized by low DHA synthetic capacity but yet high DHA needs. Comparison of the brain ethanolamine phosphoglycerols of 42 species shows an almost identical LCP pattern that, independent of EQ, is composed of about equal percentages AA and DHA.[10] A low dietary DHA intake by rats lowers DHA in the frontal cortex, downregulates DHA turnover, and increases AA turnover, which is a condition that has been related to neuroinflammation.[100] Accretion of brain DHA in newborn baboons[101] and newborn humans[102] is dependent on the postnatal dietary DHA supply during the brain growth spurt, which in humans occurs from the last trimester up to 2 years after birth. An experiment with diets varying in ALA, LA, AA and DHA administered to female mice from 3 days prior to conception showed that, in contrast to the relatively static maternal brain, the growing fetal brain is extremely sensitive to low maternal dietary DHA.[103] Many RCT aimed at the consequences of low brain DHA in human newborns have been performed. The results are at most inconclusive,[22] but various re-commendations for DHA intakes by formula and breastfed infants have been issued.[104]

Taken the above together, it seems clear that the evolution of our brain growth is unlikely to have been hampered by poor availability of dietary DHA, which is abundant in the brain of the animals that we might have consumed in the savanna, but notably the food that is available in a water-land ecosystem. Our derivation from the land-water ecosystem is strengthened by the African[10] and European[11] fossil records and the many pathophysiological consequences of a low DHA status and a low intake of brain food in general. The many indications for the exploitation of aquatic resources by early hominins date back as far as 2.3-2.0 million years ago in Semliki River, Zaire.[11] Collection of aquatic foods is still daily practice in East-Africa and picking up, clubbing, spearing or killing aquatic-animals from a distance[105] seems much easier than either scavenging or hunting game on the Serengeti plains.[10] Contrary to popular belief, our ancient ancestors did not need fishing gear to benefit from the abundance of LCPω3 and LCPω6 in such ecosystems, where it

is relatively easy to hunt and gather anything ranging from spawning (cat)fish, shellfish, crustaceans and cephalopods (lobster, crab, shrimp, squid, octopus, etc) to sea urchins, amphibians, birds and reptiles and their respective eggs.[11] All of these species ultimately receive their LCPω3 from plankton via the local food chain.[106] We seem to have experienced a diminishing consumption of food from this ecosystem since the Out-of-Africa Diaspora.[107] For instance, analysis of ^{13}C-collagen from bones[108] showed a sharp shift from a marine-based to a terrestrial-based diet in Britain at the onset of the Neolithic (4,000 years ago). Also the consumption of animal brain is conceivable, but may not solely be responsible because of its lack of many other constituents of 'brain food', notably iodine[16] Abundantly available iodine is characteristic for marine-ecosystems. The wide occurrence of iodine deficiency in people living in the inland suggests that hominin encephalization likely occurred in the land-water ecosystem. Although some traditional inland hunter-gatherers might circumvent this problem by organ consumption, including e.g. the iodine-rich thyroid of their prey, many 'modern' humans, living far from the water-land ecosystem, have abolished organ consumption and may consequently suffer from iodine deficiencies if the element is not added to table salt.

Macronutrients

Total protein intake from the presumed Paleolithic diet contributed 25-29 en% of the daily energy intake (range 8-36), which is remarkably higher than the average present day intake of 15 en% in the USA,[84] at the high range of the 10-35 en% RDA of the National Institute of Medicine,[88] somewhat lower than the data of Eaton (37 en%)[26] and comparable to the data of Cordain (30 en%, range 19-35).[27] The current estimate may be rated as a moderate-high protein diet. On a weight basis, the protein intake from a 12,500 kJ Paleolithic diet was estimated at 160-185 g/day (range 51-223) (RDA for adults 46-52 g/day{Institute of Medicine, 2005 338 /id}, of which animal protein was 108-140, and plant protein 45-54 g/day. By contrast, modern humans consume less than one-half that amount of animal protein (i.e. 64-68 g/day), and about two-thirds that amount of plant protein (i.e. 32-36 g/day) from an average diet of 10,850 kJ for men and 7,312 kJ for women.[86,109] Our models show that a Paleolithic diet results in a moderate carbohydrate intake of 39-40 (range 19-48) en%, which is similar to the 41 en% of Eaton's model.[26] The 34 en% (range 22-40 en%) intake in Cordain's model[27] was somewhat lower than current estimates, because of an assumed higher animal food intake at the expense of carbohydrate-rich plants. Current carbohydrate intakes in affluent countries average 49 en% for men and 52 en% for women,[84] while recommendations range from 40-65 en%.[88] From 1971-2000, carbohydrate intakes in the USA have increased (men: from 42 to 49; women 45 to 52 en%), at the expense of fat (men: 37 to 33; women 36 to 33 en%), saturated fat (men: 14 to 11; women 13 to 11 en%) and protein (men: 17 to 16; women 17 to 15 en%).[84] This may at least partially originate from the advice to lower fat intake, especially SAFA, and to replace by carbohydrates.[88] In our models a moderate to high 30-39 en% (range: 20-72) came from fat, which is comparable with the current Western intakes of about 33 en%,[84] at the high range of the recommended 20-35 en%,[88] higher than the 22 en% fat intake in the model of Eaton,[26] but comparable with the 36 en% (range

28-58 en%) of Cordain's model.[27]

Beasley et al.[110] showed that a carbohydrate intake in the low range of recommended (i.e. 48 en%), together with protein (25 en%) intakes at the high range of recommended and a fat intake at the recommended average (27 en%) reduces self-reported appetite, compared with diets with higher carbohydrates (58 en%) or high unsaturated fat (37 en%). In addition, compared with three other diets the low-carbohydrate/high protein 'Atkins' diet proved superior for weight loss within a one year randomized trial with overweight premenopausal women.[82] The effect is likely to be caused by better dietary adherence,[111] which was obviously superior in those receiving the high protein Atkins diet.[82,111] A high protein diet induces satiation via the anorectic hormone peptide YY[112] and satiety by its high diet-induced energy expenditure. The latter amounts to 0-3% for fat, 5-10% for carbohydrate and 20-30% for protein and is needed for intestinal absorption, initial metabolism and storage of the nutrient products that are not immediately utilized. The concomitant oxygen consumption and rise in body temperature leads to a feeling of oxygen deprivation, which promotes satiety.[113] Similar appetite-controlling effects may be expected from the Paleolithic diets (Table 5 and 6), which contain even lower carbohydrate, but higher fat, compared with the low carbohydrate/high protein diet of Beasley et al.[110] The Paleolithic diets have, however, higher carbohydrate, similar protein and lower fat, compared with the 'Atkins diet' as employed by Gardner et al.[82] The high volume of the Paleolithic diet, that is partially composed of bulky fiber in vegetables and fruits, may also enhance satiety and satiation.[114]

The weight controlling effect of a Paleolithic diet was indeed shown by Osterdahl et al.,[115] who in an uncontrolled study with healthy adults, demonstrated a decrease in weight, BMI and waist circumference after 3 weeks *ad libitum* consumption of a Paleolithic-like diet (i.e. 6,633 kJ/day; carbohydrate 40, protein 24, fat 36 en%), compared with their baseline usual diet (10,377 kJ/day; carbohydrate 54, protein 14, fat 30 en%). Similarly improved reduction of weight, BMI and waist circumference were shown in the study of Jonsson et al.,[116] who performed a 2*3 months cross-over study in type 2 diabetic patients receiving a Paleolithic diet (6,620 kJ/day, carbohydrate 32, protein 24, fat 39 en%) or a Diabetes diet (7,864 kJ/day, carbohydrate 42, protein 20, fat 34 en%). In a randomized trial in patients with ischemic heart disease plus glucose intolerance or type 2 diabetes, Lindeberg et al.[117] showed a reduced caloric intake after *ad libitum* consumption of a Paleolithic diet (5,628 kJ/day; carbohydrate 40, protein 28, fat 27 en%) as compared to an *ad libitum* Mediterranean-like Consensus diet (7,517 kJ/day; carbohydrate 52, protein 21, fat 25 en%). The studies of Osterdahl et al.,[115] Jonsson et al.[116] and Lindeberg et al.[117] suggest that the underlying reduced energy intake was due to improved appetite control. They may also have been accompanied by improved body composition, since studies on high protein intakes during body weight loss and subsequent maintenance have shown preserved or increased fat free mass at the expense of fat mass, and an improved metabolic profile.[113] Contrary to widespread belief, high protein diets do not have adverse effects upon bone mass, and especially not if the diet is also rich in fruits and vegetables.[118] They are

more likely to promote bone health and reduce the incidence of osteoporotic fractures.[113]

The current carbohydrate intake in affluent countries is not only high compared with that of our Paleolithic ancestors, but there is also a marked qualitative difference. In the era before the agricultural revolution the majority of carbohydrates were derived from fresh fruits and vegetables, together with roots and tubers, and very little from cereal grains or refined carbohydrates with high glycemic indices (e.g. highly processed grains, sucrose, fructose).[1] For hunter-gatherers wild honey provides the only type of so called 'empty calories'*, but it is only seasonably accessible and accounts for no more than 0.4-1.2 en% in the studied foragers.[3] Food products with high glycemic loads, especially when consumed in isolation, cause transient hyperinsulinemia (which is associated with CAD) and postprandial hypoglycemia (which is associated with increased hunger and lowered satiety).[119-121] There is increasing evidence that carbohydrates, especially refined carbohydrates with high glycemic indices, and food products with high glycemic loads, play important roles in the etiology of the diseases associated with the metabolic syndrome, such as diabetes mellitus type 2 and CAD.[119-121]

Evidence for the beneficial effects of Paleolithic diets may also be derived from their influence on classical CAD risk factors. The uncontrolled study of Osterdahl et al.[115] showed favorable effects on systolic blood pressure and plasminogen activator inhibitor-1, while the study of Jonson et al.[116] in type 2 diabetic patients resulted in lower HbA_{1c}, triglycerides, diastolic blood pressure, and higher HDL-cholesterol, when compared to the Diabetes diet. The trial of Lindeberg et al.[117] in patients with ischemic heart disease showed a larger improvement in glucose tolerance, independent of decreased waist circumference, for a Paleolithic diet, when compared with a Mediterranean-like Consensus diet. In an uncontrolled trial, Frassetto et al.[122] showed that 10 days consumption of an isocaloric Paleolithic type of diet (11,311 kJ/day; carbohydrate 38, protein 30, fat 32 en%): improved blood pressure, arterial distensibility, insulin sensitivity and total-, HDL- and LDL-cholesterol in healthy sedentary humans, when compared with their baseline usual diet (9,933 kJ/day, carbohydrate 44, protein 18, fat 38 en%). Importantly, there were no statistically significant changes in energy intakes, activity levels and body weight, showing that the improved CAD risk profile was unrelated to weight reduction.

Dietary cholesterol

Current recommendations for the intake of cholesterol range from 'as low as possible' to <300 mg/day.[88] Estimated intakes from the models of Eaton et al.[26] and Cordain[27] (recalculated from our data) were 480 and 830 mg/day, respectively, while the current estimates from Models 2-4 are 498, 914 and 523 mg/day. The sizeable intakes of cholesterol in Models 1 and 3 are derived from the very high amounts of cholesterol in brain (2,037 mg/100 g)[33] and marrow (119.6 mg/100 g),[123] as compared to an average of 65.9[6] in meat and 60.2 mg/100 g[33] in fish. The estimated Paleolithic cholesterol intakes are well above the present intakes of 320 mg/day in the USA[124] and the proclaimed 'high cholesterol

* High energy food without essential amino acids, essential fatty acids or micronutrients

intakes' of Japanese men (446 mg/day) and women (359 mg/day).[124] Hunter-gatherers[125] have low serum total- and LDL-cholesterol, typically ranging from 2.1-3.6 and 1.0-1.8 mmol/l, respectively, that do not increase with age.[126] The pastoral living Maasai have high intakes of fat (about 300 g/day), SAFA and cholesterol (about 600 mg/day) from both milk and meat. Yet they exhibit low serum total-cholesterol (about 3.3 mmol/L) with extensive atherosclerosis with lipid infiltration and fibrous changes of their aortas, together with intimal thickening of their coronary arteries. They are nevertheless virtually free from signs of CAD, have smaller hearts, their blood pressure exhibits only a slight tendency to increase with age, they have low body mass indices and they are remarkably fit.[127] The currently estimated cholesterol intakes by our ancient ancestors and data from the Japanese and the Maasai do not support the limitation of dietary cholesterol intake to 300 mg or 'as low as possible'. Cholesterol intakes above these recommendations might at most be disadvantageous by their interaction with other unfavorable changes in diet or lifestyle.

Fatty acids

SAFA. The average (range) SAFA intake from our reconstructed diets was 11.4 (7.2-18), 12.0 (6.9-19), 11.5 (9.9-16) and 12.0 (6.8-17) en% for Models 1-4, respectively. It is currently recommended to keep the SAFA intake 'as low as possible' and preferably below 10 en%. The association that is usually presented to illustrate that SAFA are hypercholesterolemic[88] is not only characterized by high inter-individual variation, but also by a slope that indicates that one en% SAFA increases serum total- and LDL-cholesterol by no more than 0.052 and 0.036 mmol/L, respectively.[128] It was recently concluded that there is insufficient evidence that SAFA is causally related to CAD risk.[129] Replacement of SAFA by carbohydrates at low total fat intake might even be associated with greater CAD progression.[130]

A SAFA (notably 16:0) rich diet might only be hypercholesterolemic in the context of a carbohydrate-rich diet. When carbohydrate is replaced by SAFA, the increase in HDL-cholesterol is even higher compared with replacement by MUFA or PUFA, although a significant decrease in the total/HDL-cholesterol ratio is only observed when carbohydrates are replaced by PUFA and MUFA.[131] Volek et al.[5] showed that a SAFA-rich carbohydrate-restricted hypocaloric diet (carbohydrate 12 en%, fat 60 en%, SAFA 36 g), as compared with a hypocaloric low-fat diet (carbohydrate 56 en%, fat 34 en%, SAFA 12 g; both 6,281 kJ), was not only superior in improving CAD risk factors, but also that the subjects consuming the carbohydrate-restricted SAFA-rich diet had lower SAFA in serum triglycerides and cholesterol esters. It seems that a carbohydrate-rich diet prolongs circulatory exposure to SAFA, causing a more intense interaction with Toll Like Receptors- 4 and -2,[132] and thereby the release of proinflammatory cytokines such as Monocyte Chemoattractant Protein-1 by adipocytes.[133] This mimicking of lipopolysaccharide action by SAFA may trigger the hyperlipidemia of sepsis.[134] It is increasingly acknowledged that metabolism and inflammation are intimately related.[8] The saturated lauric acid (12:0) exhibits a significant decrease of the total cholesterol/HDL-cholesterol ratio when 1 en% carbohydrates is replaced by lauric acid. Isoenergetic replacement of 10 en% of the average USA diet by carbohydrates causes a higher increase of the total cholesterol/

HDL-cholesterol ratio than butter, while coconut oil decreases this ratio.[131] It is possible that our ancient ancestors living in tropical areas at the sea experienced all benefits of the combination of moderate carbohydrate and SAFA intakes and that they had abundant access to coconut-derived lauric acid, which is not only readily absorbed but also known for its antimicrobial properties.[35]

MUFA. The MUFA intakes in Models 2 and 4 (i.e. 7.4 and 5.6) are somewhat lower and equal to those of Eaton (7.4) and Cordain (9.2),[26,27] and on the low side, when compared with the world wide intakes ranging from 8 en% in parts of China[135] to more than 20 en% in the Mediterranean.[136] The average MUFA intake in Model 3 (i.e. 13.0 en%) is similar to the 13 en% in the USA[81] while the 18.5 en% from Model 1 is intermediate to this 13 en% and the high intakes in the Mediterranean. Populations consuming high MUFA diets show low incidence of CAD.[137] However, although Japanese have low (9 en%) MUFA intakes,[135] they also have low CAD risk.[138] When replacing SAFA by MUFA (11 en%) or carbohydrate (20 en%), MUFA provided a greater risk reduction in CAD than did carbohydrate,[139] while replacement of 1 en% carbohydrates with cis-MUFA caused a steep decrease in the total cholesterol/HDL-cholesterol ratio.[131] Taken together it seems that MUFA are notably beneficial at isocaloric low-moderate carbohydrate intakes.

ALA and LA. In our models the median ALA consumption and its range (Table 5**)** indicate that the mixing of fish and the introduction of selective consumption of meat into the savannah-derived Paleolithic diets of Eaton and Cordain[1,26-28], increases the ALA intake to an average of 3.7-4.7 (range 2.1-5.8) en%. The models also show consistently low LA intakes (2.3-3.6, range 1.7-6.2 en%). After employment of the protein constraint it proved unnecessary to additionally employ the 1.0 en% LA constraint, since all of the remaining options within the 4 foraging strategies provided over 1.0 en% of LA.

The average daily intake of ALA in British omnivores was 0.4 (1.3 g/day) and 0.7 en% (2.2) for vegans,[140] which are both remarkably lower than the ALA intakes from the current and earlier Paleolithic diets of Eaton and Cordain,[26,27] It is possible that the easily recycled[141] dietary ALA constituted an important precursor for the synthesis of SAFA, MUFA, cholesterol and ketone bodies for energy generation. The higher ALA intakes, as compared to LA, might additionally explain why ALA is β-oxidized twice as fast as LA.[142] High ALA intakes from a Paleolithic diet might also be in line with the indications that ALA plays an important role in CAD prevention.[143] Anti-inflammatory effects of a high ALA-intake include reductions of C-reactive protein, soluble intercellular adhesion molecule 1, soluble vascular adhesion molecule 2, IL-6, IL-1beta and TNF-alpha.[143,144] Inconclusive evidence for the role of ALA in CAD[129] might be explained by low intakes of ALA in combination with relatively high LA intakes and would be consistent with the stronger evidence linking increased ALA intake to low CAD risk, as observed for subjects with low fish intakes.[145] The current models with ALA/LA ratios ranging from 0.61-1.79 g/g (Table 5) reassert the unfavorable ALA/LA ratio in the Western diet, while such ratios were also used in many, if not all, intervention trials.

The LA intake of the models did not exceed 6.2 en%. These outcomes are comparable with the earlier data of Eaton[26] and Cordain.[27] It is currently advised to augment the LA intake to 5-10 en%,

since it was concluded that aggregate data from RCTs, case-control and cohort studies, and long-term animal feeding experiments indicated that these intakes reduce CAD risk.[146] This, recently reinforced, advice by the AHA has taken the daily LA intake in Western countries to 4-9 en%.[71] It also caused an increase of the breast milk LA content to >15 g% in many Western countries,[71] which is significantly higher compared with the 4.2-5.2 g% LA in the milk of traditionally eating Tanzanian populations.[35] Our data indicate that such an LA status can not be achieved without the consumption of refined vegetable oils. These have never been part of the diet on which our genome has been evolved to what it currently is. Correction of our current high CAD risk, by the introduction of nutrient intakes that have never been reached in the past, harbors the risk of introducing new imbalances. Employing the Hill-criteria for causality, Mente et al.[129] recently concluded that there is insufficient evidence that PUFA is causally related to CAD risk. In addition it seems that despite the ensuing recommendation to consume 5-10 en% LA, these RCT with positive outcomes have been conducted at 11-21 en% LA.[146] Moreover, these high LA intakes, which were even combined with increased intakes of ALA, fish or cod liver oil, replaced SAFA in the included trials.[146] High intakes of LA, but not AA,[147] have been related to the etiology of ulcerative colitis,[148] while the LA intake was positively associated with a risk of severely depressed mood[149] and homicide.[21] Studies with human endothelial cells suggest that LA might promote inflammation through activation of NFκB, and increased production of TNF-α, IL-6 and other inflammatory mediators.[150] The high prevalence of CAD, hypertension, type 2 diabetes mellitus and obesity in Israel, also referred to as the 'Israelian paradox',[151] might be related to the nationwide 8-12% higher PUFA intakes in Israel, as compared to other Western countries. Ailhaud et al.[71] considered the decreasing dietary ALA/LA ratio, due to both an increase in LA and decrease in ALA, in Western diets as a possible cause of obesity. Finally, it has become clear that the current high LA intake causes inhibition of the conversion of ALA to EPA and DHA, and thereby contributes to our currently low LCPω3 status.[152]

LCP. In Models 2, 3 and 4 the average sum of the intakes of the fish oil fatty acids EPA and DHA were 6.1 (range: 0.88-28.3), 5.8 (range: 1.38-11.6) and 14.2 (range: 5.34-28.3) g/day. Because of the inclusion of food from the water-land ecosystem these figures were much higher than those of Eaton[26] and Cordain.[27] The calculated average AA intakes of 3.65, 2.84 and 5.46 (ranges: 1.69-10.7; 1.15-4.61; 2.14-10.7) g/day, respectively, are also considerably higher compared to the 1.81-2.41 (range: 1.15-2.77) g/day of the savannah models.

The high LCPω3 intake (median 7.64 g/day) in Model 2 is about half the intake of Inuit who have lifetime consumption of about 14 g LCPω3/day,[17] while the 17.0 g LCPω3/day of Model 4 is even somewhat higher. The similarity between the milk LCPω3 contents of Tanzanian mothers with high daily consumption of fresh water fish from Lake Victoria[35] and the milk LCPω3 contents of Canadian Inuit[153] adds to this notion. Both model outcomes are also in line with estimates of Broadhurst et al.[12] who calculated a daily intake of 29 g fish oil from a 9,196 kJ diet by current native Africans living on the shore of Lake Malawi. With a 15 g% average EPA+DHA content of the local fish, this would imply an intake of at least 6 g LCPω3 per day, which is remarkably comparable with the intakes from

Models 2 and 3. Crawford et al.[154] estimated the daily intake of contemporary populations living at East African lakes (Lakes Nyasa and Turkana) at 1-4 g LCPω3 and 0.5-1.0 g AA, respectively. All of the above data suggest that in the Paleolithic era the intakes of EPA plus DHA greatly exceeded those currently recommended in Western societies (450-500 mg/day[155,156]), while also the AA intake was much higher than at present (about 200 mg/day[157]). Of interest is the lower (EPA+DHA)/AA ratio in African fish (2.67 g/g) and consequently in the fish-based Paleolithic diets (1.82-2.60 g/g), as compared to Northern latitude fish (7.48 g/g in cold water fish; 8.74 g/g in European fish) (Supplemental Table 1). It seems that many, if not all, intervention trials have been conducted with high (EPA+DHA)/AA ratios and certainly with relatively low absolute amounts of (EPA+DHA), while the intake of AA is usually ignored.

Our finding of high LCP intakes compared with the parent precursors LA and ALA, and the knowledge that about 75 g% of dietary LA and ALA is fully β-oxidized, even under extreme dietary LCPω3 deficiency,[141] put the FADS polymorphisms,[158] the concept of 'essential fatty acid deficiency' and also the ω3/ω6 ratio into different perspectives. In contrast to the present, both the FADS polymorphisms and our difficulty to synthesize DHA might have been unimportant in the past. The chain elongation/desaturation pathway might not have been used at all and LA and ALA deficiency might never have occurred. LA deficiency may be defined clinically in terms of symptoms such as a scaly dry skin and reproductive failure,[159] and ALA deficiency in terms of numbness, paresthesia, weakness, pain and blurred vision,[160] while these deficiencies are biochemically characterized in terms of the accumulation of Mead acid[161] or an increased Mead acid/AA ratio.[142] Neither symptoms, nor Mead acid accumulation will occur when the intakes of AA and DHA are high, since these would inhibit FADS1 and 2 activities. Also the specific function of LA in the synthesis of skin ceramides might be conserved and thereby skin's water barrier function, since dietary AA might in such circumstances become retroconverted to LA.[162] Retroconversion of DHA to EPA[163] and possibly to ALA may also have taken place. Since under these conditions neither LA nor ALA would be essential, it was suggested[142,164] to consider AA and DHA as the genuine essential fatty acids. It would also imply that the presently employed constraint of 1.0 en% LA would be superfluous and that the vigorously debated 'healthy' dietary ω3/ω6 ratio[107] and the currently depicted (EPA+DHA)/AA ratio might have been unimportant in the past. AA, EPA and DHA might under these circumstances not compete with each other, but rather jointly compete with other fatty acids causing full saturation of tissues with LCP. This is currently not the case. For instance, Hsieh et al.[101] showed that raising the milk DHA content from 0.3 to 1.0 g%, both at 0.7 g% AA, caused an increase of DHA in virtually all investigated brain regions of the newborn baboon without affecting their AA contents. This suggests that newborns in Western countries have low brain DHA, since many of their mothers have milk DHA contents in the 0.3 g% range.[34] Finally, Liou and Innis[152] recently showed that minimum LA requirements for AA synthesis are below 3.8 en% and suggested that the encountered two fold inter-individual variance of AA status might be due to FADS polymorphism. The estimated AA intakes by our Paleolithic ancestors suggest that neither of these problems might have been of

importance in the past.

The suggested high dietary intakes of LCP, notably those of AA, raise questions regarding their toxicities. In a recent study it was concluded that estimated DHA intakes of up to 315 mg/day by 1-6 months infants are safe and that no consistent adverse effects in platelet function, lipid levels, in vivo oxidation parameters, glycemic control, or immune function have been observed in adults taking up to 7.5 g DHA per day.[165] Inuit[17,153] and the many fish eating populations in other countries, including Africa,[34,35] are living testimony of this thesis. AA is, however, invariably feared for its role in coagulation and inflammatory reactions. A human feeding study with 6 g AA/day (supplemented as ethyl ester) was terminated because of a marked increase in *in vitro* platelet aggregation.[166] In subsequent well-controlled studies, Nelson and co-workers observed no changes in blood coagulation, thrombotic tendencies[167] or immune functions,[168] but also a small increase in neutrophil count and immune response to influenza vaccine,[169] together with increases in thromboxane, PGI_2,[170] PGE_2 and LTB_4[168] in healthy male adults consuming 1.5 g AA for 50 days. They attributed the near absence of changes in the immune functions tested to derive from the opposing effects of prostaglandins and thromboxanes. Recently, Kusumoto et al.[171] showed that 4 weeks supplementation of 840 mg AA per day did not affect metabolic parameters or platelet function. Moreover, the 'pro-inflammatory' AA is also needed for the synthesis of AA-derived lipoxines, which are pro-resolving and mediate the switch to the synthesis of the anti-inflammatory resolvins and protectins synthesized from EPA and DHA.[172] It should finally be noted that all these studies were performed with high AA, low LCPω3, supplements. In our models, the increase in EPA+DHA is more pronounced compared to the increase in AA [see ratio (EPA+DHA)/AA in Figures 2 and 3], with increasing LCP intakes. Other studies relating AA with or without concomitant EPA+DHA intakes to eicosanoid production showed that dietary AA enhanced in vivo eicosanoid production,[173] while even low doses of AA were able to reverse EPA+DHA induced decreases in PGI_2 and TxA_2 production.[174] Although short term AA-rich meat diets did not affect platelet aggregation, TXB_2, PGI_2 or TXA_2 production,[175] O'Dea et al.[176] showed a rise in bleeding time after consumption of LA and AA-rich meat and fish. Together, the health effects of AA are still controversial and there is as yet insufficient evidence to decide whether high AA, with or without concurrent high LCPω3 intakes, are harmless or beneficial,[150,177] but high intakes of AA clearly need caution. However, it is likely that at high dietary AA and DHA intakes, the surpluses will for a great deal be used for retroconversion, energy generation, or both, although to our knowledge no hard data are available in support of this notion.

Limitations

The outcome of our models should be viewed upon as indicative for the range of the dietary compositions of our ancestors, since we have obviously no hard data on their foraging strategies and the nutrient compositions of the plants and animals that they consumed. Our data rely on the comparability of the compositions of contemporary foods with the foods available in the Paleolithic and on the representativeness of the foods that have been analyzed. Whether our earliest ancestors

employed cooking remains controversial, but its employment would increase the digestibility of certain plant foods and consequently the availability of certain nutrients. While for fish species an increasing amount of literature on energy density, macronutrient content and fatty acid composition is available, comparable literature on wild animals and plants is scarce or inaccessible. With respect to East-African plant specimen the literature provides even less information. For example, the plant AA contents are derived from a single publication and those analyses were not confirmed by mass spectrometry. Despite the even higher AA content of seaweed and insects, the AA contents of many plant foods are probably lower, and their AA contents certainly warrant further studies. In other words, a better approximation of the Paleolithic diet would be possible when more complete information on fatty acids in African nuts, fruits, roots, bulbs, gums and tubers would become available. There is no evidence that our ancestors have used fishing gear prior to 300,000 years ago.[11] However, there is circumstantial evidence that fish up to 1 meter in length were caught bare handed, when spawning during the rainy periods or when trapped in pools during drought.[9] Nevertheless, the current use of the fatty acid composition of East-African fish might not be appropriate, and in reality better be described by some combination of the fatty acid compositions of a variety of animals living in the land-water ecosystem, who ultimately receive their LCP from plankton. Apart from fish however, we could not recover any data on the fatty acid composition of such African species. Taken together, an increasingly complex multidimensional model differentiating between African plant foods (seaweeds, nuts, seeds etc), land animals (all edible organs) and marine animals (fish, shellfish, eggs) and in which all separate intakes and nutrient compositions are varied within their most realistic ranges would certainly improve the current estimates. Finally, due to differences in environmental circumstances, Paleolithic and modern humans might have (had) different benefits from the intake of fatty acids, such as AA. High AA intakes might confer unfavorable effects in Western societies, where morbidity and mortality stem mostly from chronic diseases with inflammation and thrombosis, but might have been favorable to Paleolithic humans who were mainly confronted with morbidity and mortality from infection and trauma. The latter suggests that AA might have conferred antagonistic pleiotrophy in the past.

CONCLUSIONS

We found that the macronutrient composition of the presumed Paleolithic diet averaged 25-29 (range 8-35) en% from protein, 39-40 (19-48) en% from carbohydrate and 30-39 (20-72) en% from fat. These data imply that Paleolithic diets provided moderate-to-high protein and fat intakes, and moderate carbohydrate intakes. The fatty acid composition was moderate-to-high in MUFA and PUFA, but relatively high in SAFA. The PUFA were notably high in ALA, LCPω3 and LCPω6, but low in LA, compared to current Western intakes and recommendations. With the previous limitations in mind, the current data reflect the nutritional balance on and selection pressure under which our genome evolved. Our models reveal consistent differences between estimated Paleolithic macronutrient and fatty acid intakes and those found in contemporary Western diets as well as recommendations.

Together with other human caused environmental changes, these disparities are likely to play an important role in the etiology of Western disease. For example, the hypercholesterolemic effect of carbohydrates, the positive relation between protein intake, satiety and satiation as well as the many beneficial effects attributed to LCP suggest a beneficial role for the consumption of Paleolithic-like diets. These diets do not as much affect our life expectancy, but rather our years in good health. Interestingly, many of the dietary disparities are at present heavily debated, suggesting that both the approaches via intervention trials and evolutionary medicine identify critical dietary factors that are important to current Western diseases. We suggest that the present data represent a unique and powerful rationale for the design of future intervention studies.

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Addendum and units

'Plant' refers to any edible component from the kingdom of *Plantae* and *Fungi,* while 'animal' refers to edible components from the kingdom of *Animalia*. For meat consumption, only 50 g% of the total carcass weight was considered as edible material.[62] The weights of the consumed organs are therefore presented as a percentage of the total edible material, i.e. 3.8 g% for the liver, rather than as a percentage of total carcass weight (i.e. 1.9 g% for the liver). Plant food and fish were considered to be consumed completely. Fat contents are consistently presented as g/100 g edible material (g%). Fat contents refer either to the specific fat% of a particular organ (e.g. the brain) or the fat% of all edible material (i.e. the fat% of all combined edible material, including lean meat, brain, liver, bone marrow and adipose tissue. Plant/animal and meat/fish ratios and ratios between skeletal muscle, brain, bone marrow, liver and adipose tissue are given in energy% (en%). Fatty acid compositions are given in g/100 g fatty acids (g/100 g, g%). Organ weight contributions to selectively and non-selectively consumed meat are presented as g/100 g meat.

Supplemental Table 1. Energy density. lipid and fatty acid contents of plant foods. savannah foods (meat: muscle. brain. bone marrow. liver and adipose tissue) and aquatic foods (fish [salt/fresh water. cold/tropical water. particular African lakes and rivers]. eggs (from reptiles. birds. fish). crustacaeans. cephalopoda. mammals. reptiles and birds (all muscle meat. for some liver and adipose tissue).

	Energy	Lipid	ALA	EPA	DPA	DHA	LA	AA	SAFA	MUFA	PUFA	LA/ALA	(EPA+DHA)/AA	reference
	(kJ/100 g)	(g/100 g)	g%	g%	g%	g%	g%	g%	g%	g%	g%	g/g	g/g	
Plant Food														
Plant														
Africa (45)														
Terrestrial leaves (14)		0.83	41.1				12.6		37.0	7.05	53.8	0.31		54-55
Seeds (11)		14.5	3.42			0.42	20.1		18.5	19.5	24.1	5.86		54
Nuts (4)		46.4	0.18				2.47		77.3	10.6	2.65	13.69		51.55
Roots/tubers (14)	325	1.66	9.02				22.5		35.3	32.3	31.5	2.49		51-52.55
Fruits (2)		9.86	1.01				13.7		34.5	50.8	14.7	13.52		51.55
World (788)														
Terrestrial leaves (20)	469	0.38	32.9	0.61	0.15	0.26	12.3	0.64	24.3	9.77	50.1	0.38	1.36	26-50
Vegetables (768)	699	4.99												49
mean	**364**	**7.10**	**26.1**	**0.61**	**0.15**	**0.32**	**14.0**	**0.64**	**29.2**	**12.5**	**42.3**	**0.54**	**1.45**	
Savannah Food														
Muscle														
Africa (11/51)	469	2.87	4.24	1.34	2.92	0.37	20.72	6.40	39.16	21.58	36.34	4.88	0.27	55.64-68
North-America (4/42)	456	2.50	3.39	2.16	2.88	1.31	13.76	6.52	34.78	24.42	26.55	4.06	0.53	47
Asia (2/2)	398	0.95	2.60				15.00		29.65	20.35	30.00	5.77		69.189
Australia (20/46)	419	1.51	2.26	1.32	2.23	1.89	22.87	10.41	30.90	25.39	43.39	10.14	0.31	69-70.189
mean (35/128)	**436**	**1.96**	**3.12**	**1.61**	**2.68**	**1.19**	**18.09**	**7.78**	**33.62**	**22.94**	**34.07**	**6.21**	**0.37**	
Brain														
North America (3/46)	528	9.10	0.20	0.04	0.63	9.26	0.69	5.74	31.78	27.63	21.98	3.51	1.62	33.47
Marrow														
Africa (5/17)	2044	51.0												45
North America (5/53)			1.59	0.00	0.00	0.00	2.40	0.00	23.23	64.06	6.04	1.51		47

Supplemental Table 1. *Continued*

	Energy (kJ/100 g)	Lipid (g/100 g)	ALA g%	EPA g%	DPA g%	DHA g%	LA g%	AA g%	SAFA g%	MUFA g%	PUFA g%	LA/ALA g/g	(EPA+DHA)/AA g/g	reference
Eaton (3)			0.86	0.08	0.06	0.07	7.08	0.16				8.23	0.94	26
mean (13/73)	**2044**	**51.0**	**1.23**	**0.04**	**0.03**	**0.04**	**4.74**	**0.08**	**23.23**	**64.06**	**6.04**	**4.87**	**0.94**	
Liver														
Africa (2/2)	666	7.10	4.55	0.95	4.85	2.00	13.50	9.35	36.30	22.35	36.95	2.97	0.32	68.181
Australia (4/10)	595	6.34	5.08	0.86	1.25	1.20	17.34	9.45	34.29	29.98	36.76	3.42	0.22	70
mean	**631**	**6.72**	**4.81**	**0.91**	**3.05**	**1.60**	**15.42**	**9.40**	**35.29**	**26.16**	**36.86**	**3.19**	**0.27**	
Adipose tissue														
Africa (7/18)	3120	82.3	4.19				4.87		51.05	27.19	12.76	1.16		63.66-67
Australia (5/11)	3262	86.1	5.63	0.01	0.14	0.05	9.63	0.25	37.41	46.69	15.90	1.71	0.26	70
North America (3/41)			1.27		0.01		1.85	0.01	63.67	28.88	3.96	1.45		47
mean (15/70)	**3191**	**84.2**	**3.56**	**0.01**	**0.07**	**0.04**	**5.41**	**0.21**	**51.15**	**35.17**	**9.53**	**1.52**	**0.25**	
Aquatic Food														
Freshwater fish														
Africa (47)														
Lake Kitangiri (4)			2.92	3.31	3.77	9.17	3.69	10.03	38.63	20.94	40.43	1.26	1.25	34-35
Lake Nyassa (15)	507	4.53	1.61	3.28	3.65	14.11	3.07	8.45	35.80	21.18	43.02	1.90	2.06	34-35.72
Lake Turkana (2)	431	2.45				16.05		7.65					2.10	10.12
Lake Victoria (28)			1.39	4.33	4.68	16.99	2.31	5.99	36.93	22.00	41.07	1.66	3.56	34-35
The river Nile (4)			0.98			12.90	2.28	7.95				2.33	1.62	73
mean	**469**	**3.49**	**1.73**	**3.64**	**4.03**	**13.84**	**2.84**	**8.01**	**37.12**	**21.37**	**41.50**	**1.64**	**2.18**	
Asia (20)		7.36	0.42	0.61		0.84	10.94	2.80						188
Australia (6)	536	5.30		2.68		5.75	3.62	2.68					3.14	193
Europe (6)	385	1.19	1.56	6.14		13.55	3.53	8.28				2.26	2.38	187
North America (22)	465	3.33	3.15	7.22		13.97	3.28	5.66				1.04	3.74	178.183
South America (24)	511	4.60	4.31	3.86		6.66	6.01	4.32				1.39	2.43	179

Supplemental Table 1. *Continued*

	Energy (kJ/100 g)	Lipid (g/100 g)	ALA g%	EPA g%	DPA g%	DHA g%	LA g%	AA g%	SAFA g%	MUFA g%	PUFA g%	LA/ALA g/g	(EPA+DHA)/AA g/g	reference
mean	**474**	**3.61**	**3.01**	**4.98**		**9.98**	**4.11**	**5.24**				**1.36**	**2.86**	
mean all freshwater	**473**	**4.13**	**2.36**	**4.16**		**9.23**	**4.90**	**5.29**				**2.07**	**2.53**	
Saltwater fish														
Africa (21)			0.60	6.57	3.18	19.33	1.81	9.67	37.21	15.49	47.30	3.01	2.68	35
Asia (33)	490	4.08	0.81	7.83		19.53	9.02	4.71				11.14	5.80	182.186
Australia (90)	406	1.73	1.12	5.66		23.13	1.26	6.75				1.13	4.27	70.73.180.184.193
Carribean (12)			0.36	5.64		26.58	1.13	6.54				3.18	4.93	34
Europe (62)	477	3.66	1.37	7.78		18.23	2.63	2.97				1.92	8.74	73.187.190
South America (26)	544	5.56	2.15	9.56		21.81	1.74	5.22				0.81	6.01	192
mean	**479**	**3.76**	**1.07**	**7.17**		**21.43**	**2.93**	**5.98**				**2.75**	**4.79**	
mean fresh- and saltwater	**476**	**3.94**	**1.72**	**5.67**		**15.33**	**3.92**	**5.63**				**2.28**	**3.73**	
mean African fish	**469**	**3.49**	**1.06**	**5.18**	**3.76**	**17.40**	**2.19**	**8.45**	**37.01**	**18.59**	**44.40**	**2.07**	**2.67**	
Cold water fish (13)	377	0.91		9.91		36.44	0.45	6.20					7.48	73
Tropical water fish (27)	394	1.44		4.14		28.24	1.64	6.32					5.13	73
Not included foods														
Insects (11)		15.0	2.0	0.07	0.0		18.1	1.77	33.4	49.91	16.25	8.87		60-61
Not included aquatic foods														
Seaweed/grass/algae (13)		1.40	13.5	9.60		5.00	5.8	4.50	45.8	11.1	43.5	0.43		56-59
Fish Roe (8)		7.55		7.68		17.03	1.70							73.193
Crustaceans/Cephalopods (43)	398	1.55	1.82	12.84		16.83	2.70	4.60				1.48	6.45	70.73.182.185.193
Mammals/Reptiles/Birds (5)		1.26	4.10	2.02		8.33	11.46	15.62				2.80	0.66	70
Eggs (Reptile/Bird) (3)		7.70	0.26	1.04		2.72	1.76	3.04				6.92	1.23	70.191
Liver (Mammals/Fish) (2)		34.55	1.75	4.05		5.75	3.00	13.80				1.71	0.71	70.191
Adipose tissue (Birds/Reptiles/Fish) (4)		83.33	4.45	2.45		2.90	7.85	7.53				1.76	0.71	70.191

Legend for Supplemental Table 1.

Data for this database were adapted from references [2,26,34,35,45,47,49-61,63-68,70,72,73,178-193] *For abbreviations: see Section Abbreviations*

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CHAPTER 3.1

High contents of both docosahexaenoic and arachidonic acids in milk of women consuming fish from lake Kitangiri (Tanzania).

Targets for infant formulae close to our ancient diet?

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ABSTRACT

Current recommendations for arachidonic (AA) and docosahexaenoic (DHA) acids in infant formulae are based on milk of Western mothers. Validity may be questioned in view of the profound dietary changes in the past 100 years, as opposed to our slowly adapting genome. Hominin evolution occurred in the proximity of East-African freshwater lakes and rivers and early *homo sapiens* had higher intakes of AA and DHA from a predominantly lacustrine-based diet. In search of milk AA and DHA contents of our African ancestors we investigated the milk of 29 lactating women living in Doromoni near lake Kitangiri (Tanzania). They consumed sunflower oil-fried local fish as only animal lipid sources, maize and local vegetables. AA and DHA contents of Doromoni milk may be close to that of early *homo sapiens*, because of the similarity of their life-long consumption of East-African lacustrine-based foods. Human milk fatty acid relationships from our historical worldwide database and the literature revealed that disparities between the Doromoni diet and the presumed ancient diet (i.e. higher carbohydrate and linoleic acid intakes) are unlikely to affect milk AA and DHA contents. Doromoni milk had high contents of AA (median 0.70 mol%), DHA (0.75) and eicosapentaenoic acid (EPA, 0.17), and low AA/DHA ratios (median 0.91; 0.55-2.61). This tracks down to consumption of fish with high AA and DHA contents, and AA/EPA ratios. We conclude that the milk AA, DHA and EPA contents of Doromoni women might provide us with clues to optimize infant formulae and perhaps the milk of Western women.

INTRODUCTION

The long chain polyunsaturated fatty acids (LCP) docosahexaenoic (DHA, 22:6ω3) and arachidonic (AA, 20:4ω6) acids are major constituents of brain phospholipids.[1-3] Many studies have shown their prenatal importance and the postnatal importance of notably DHA in early human (brain) development.[4-6] The addition of both DHA and AA to formulae for premature infants and to lesser extent term infants, is nowadays widely accepted. Current recommendations for their addition to formulae are largely based on the concentrations observed in milk from Western mothers. Typical recommendations would be AA≥0.40%, DHA≥0.35% for prematures and AA≥0.35%, DHA≥0.20% for terms[7] or AA=0.50%, DHA=0.20%, eicosapentaenoic acid (EPA; 20:5ω3) <0.10%.[7] However, the worldwide human milk fatty acid composition is subject to high interindividual biological variation, mostly because of different maternal diets. EPA (interindividual biological variation: 100%) and DHA (68%), exhibit the highest biological variation, whereas palmitic acid (16:0; 12.7%) and AA (28%) rank among the lowest. Consequently, one may question the basis of current recommendations, and notably whether milk from Western mothers, if any, should serve as the gold standard.[8]

Adequate amounts and ratios of LCP in newborn nutrition are important, since LCP do not only serve as structural building blocks and precursors of eicosanoids, but are increasingly recognized to impact the expression of our genome in their capacity to act as activators or repressors of a number of nuclear receptors that function as ligand-activated transcription factors.[9-12] Our genome has largely evolved on a hunter-gatherer diet and it is unlikely that our genes have completely adapted to the change of diet since the agricultural revolution some 10,000 years ago and notably the rapid dietary changes that occurred in the past 100 years.[13,14] There is ample evidence that hominin evolution took place on the shores of freshwater lakes, rivers and the sea in (East) Africa and that early *homo sapiens* had higher intakes of LCP from a predominantly lacustrine- and marine-based diet that is rich in both LCP of the ω3 series (LCPω3) and AA.[15-17] In contrast, most of the fish consumed in Western countries, especially fatty fish, is rich in DHA and to lesser extent EPA, but relatively poor in AA.[16,18] Fish consumption in Western countries, e.g. The Netherlands, is limited as compared with e.g. some Caribbean countries, which explains the high worldwide biological variation of DHA and EPA.[8] The resulting low LCPω3 status in Western countries is related to coronary heart disease (CHD) and mental and psychiatric disturbances,[19] and randomized controlled trials with LCPω3 showed reduced mortality from CHD and a reduction of schizophrenic symptoms by EPA supplements.[20] Analogously, low maternal LCPω3 status gives rise to low newborn LCPω3 status,[21] which will be maintained by low milk LCPω3 concentrations with as yet unknown (long term) consequences.

In contrast to LCPω3, there seems to be less doubt regarding recommendations for the formulae AA content. Vegetarians and vegans have similar human milk AA contents as compared to omnivores and supplementation of lactating women with 300 mg AA for one week did not augment their milk AA content.[22] Consequently, as also reflected by its relatively low worldwide biological variation,[8] the milk AA content seems less dependent on the dietary AA intake, which might imply that it can safely serve as a basis for infant formulae recommendations. AA and LCPω3 are on the other hand

known for their competitive behavior, but collaboration has also been noticed.[23,24] It seems that in reality little is known on the LCPω3-LCPω6 interaction in terms of competition or collaboration and that from a practical point of view the most valuable data for recommendations should come from the study of milk from women who consume our presumed ancient diet, or a diet that contain the LCPω3 and AA-rich East-African freshwater lake fish as the major lipid source.

We hypothesized that human milk from hunter-gatherers living in the vicinity of the East-African lakes would provide us with the closest as yet available information on the evolutionary background of the human milk LCP contents. A possible study group would be the Hadzabe people living near lake Eyasi (NE Tanzania), but these are unfortunately not easily accessible for such studies. We therefore decided to investigate lactating women who live in Doromoni near lake Kitangari (Tanzania). These women consume sunflower oil-fried fish as the only source of animal lipid and protein, together with 'ugali' (maize porridge) and various types of local vegetables. A high carbohydrate intake is known to rapidly increase the milk medium chain fatty acid (MCSAFA; 6:0 up to 14:0) content,[25-27] but has negligible effect on the milk LCP content. This was apparent from the human milk fatty acid database of a variety of different countries that we[8,28] have compiled during the past 25 years (further referred to as our 'world' human milk fatty acid database). As a control group we selected women living in Mwanga (about 55 km in the flat-land East from Doromoni), who predominantly eat vegetarian diets. The milk fatty acid data from Doromoni were also compared with historical data from the Netherlands and the Caribbean. In addition, we sampled the fish from various Tanzanian freshwater lakes and compared their fatty acid compositions with those of counterparts from Caribbean waters (i.e. Curaçao, Netherlands Antilles) and the North Sea (i.e. the Netherlands).

Materials and methods

Milk was sampled from women attending local dispensaries in Doromoni and Mwanga (Tanzania). Women were eligible to participate if they were apparently healthy, well nourished as derived from anthropometric data, and had delivered an apparently healthy child more than 10 days prior to their visit. Anthropometric data were recorded. Data on parity, number of living children, lactation duration, diet and tribe were obtained from their medical records or from a translator-assisted interview by one of us. The characteristics of the women are depicted in **Table 1**. The study was approved by the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/145, Dar es Salaam, dated June 16, 2003) and was in agreement with the Helsinki declaration of 1975 as revised in 2000.

About 5 mL of milk was collected by manual expression. Following gentle mixing, 100 μL was immediately pipetted into a teflon-sealable tube that contained 1 mL methanol-6 mol/L HCl (5:1 v/v) and 1 mg butylated hydroxytoluene (BHT, antioxidant). Fatty acids are stable in this mixture at room temperature for months. Fish were caught by line or purchased from local fishermen or in markets at lakes Kitangiri, Victoria and Nyasa (also named lake Malawi). An approximately 1 cm^3 sample of the edible proportion was taken from a site just below the dorsal fin and just above the backbone.

Table 1. Subject characteristics

	Doromoni (n=29)	Mwanga (n=26)
Age (years)	24 (16-36)	24 (18-42)
Length (m)	1.56 (1.43-1.72)	1.58 (1.43-1.68)
Weight (kg)	55.5 (41-70)	55.5 (40-74)
BMI (kg/m2)	22.7 (18.2-30.3)	22.1 (17.9-28.8)
Upper arm circumference (cm)	27.2 (23.0-32.0)	25.3 (22.0-32.0)
Parity (number)[a]	2.0 (1-7)	2.5 (1-10)
Children alive (number)[a]	2.0 (1-6)	2.5 (1-7)
Duration of lactation (days)[a]	107 (13-380)	104 (23-405)
Diet		
Days of fish/week(%)	7 (7-7)	0.5[b] (0-5)
Days of meat/week(%)	0 (0-0)	0.9 (0-3)
Tribe (%)		
Iramba	100	27
Barabaik	0	8
Iraki	0	38
Unknown	0	27

Data are means (range) unless otherwise indicated.

[a]. Median (range)

[b]. 3 women reported to eat 2. 2 and 5 days fish/week. respectively. their milk DHA ranged from 0.17-0.26 mol%

The sample was transferred to a teflon-sealable tube that contained the same preservation/ transmethylation mixture (see above). The local sunflower oil was also sampled and preserved for further analysis of its fatty acid composition. All samples were kept at room temperature and in the dark until transportation to the Groningen University Hospital (the Netherlands) for analysis of their fatty acid contents.

Analyses of fatty acid methyl esters (FAME) were performed with capillary gas chromatography following the addition of a series of odd-chain numbered fatty acid internal quantification standards (5:0 up to 17:0), transmethylation and extraction of FAME according to previously reported methods.[29] Long-chain fatty acids (≥16:0) were quantified on the basis of the added 17:0. Medium chain fatty acids (6:0 up to 14:0) were quantified with use of 5:0 - 15:0 as internal quantification standards.[30] Fatty acid compositions were expressed in mol% and ratios in mol/mol. Between-group differences were tested with the Mann-Whitney U-test at $p<0.01$ (i.e. with correction for type 1 errors, according to Bonferroni). We used multiple linear regression to investigate whether the 'world' human milk fatty acid database and the fatty acid data bases of Dormoni and Mwanga exhibited differences in the relations between MCSAFA on the one hand and oleic acid (18:1ω9), linoleic acid (18:2ω6), LCPω3 and LCPω6 on the other hand. The variables were log-transformed to obtain linear relations, if necessary.

RESULTS

Table 2 shows the fatty acid compositions of milk from women living in Doromoni and Mwanga, as compared with historical data from The Netherlands and the Caribbean.[8] The latter were obtained with use of the same analytical method. The LCPω3 (notably EPA, 22:5ω3 and DHA) and LCPω6 (notably AA, 22:4ω6 and 22:5ω6) contents of milk from women living in Doromoni was higher and the ω9 (notably 18:1ω9) content was lower than corresponding contents in the Netherlands, Caribbean and Mwanga. Doromoni milk had higher MCSAFA (notably 12:0 and 14:0) content compared with The Netherlands, but similar MCSAFA contents as in the Caribbean and Mwanga. The milk polyunsaturated fatty acid (PUFA, notably 18:2ω6) content in Doromoni was higher than that in the Netherlands and Caribbean, but similar to that in Mwanga. The Doromoni milk had the lowest AA/DHA ratio and the highest EPA/DHA ratio. The milk from mothers living in Mwanga had higher LCPω3 content compared with the Netherlands, but its DHA content was lower compared with both the Netherlands and the Caribbean. Its MCSAFA, AA, 22:4ω6 and 22:5ω6 contents were higher than in the Netherlands, but these contents were similar to those in the Caribbean.

Figure 1 and 2 depict the relations between DHA and AA (Figure 1) and EPA and AA (Figure 2) in milk of mothers from Doromoni and Mwanga, as compared with historical data from our 'world' human milk fatty acid database.[9]

The contents of AA, DHA and EPA in milk from Doromoni proved exceptionally high, both when compared with Mwanga and our 'world' database. In contrast to the relation between milk DHA and AA in Doromoni, our data for the 'world' suggested that the milk AA content decreased when milk DHA exceeded 0.6-0.7 mol% (Figure 1).

Seventeen mothers (58.6%) from Doromoni had milk DHA and AA contents above 0.65 and 0.61 mol%, respectively, as compared with none of the mothers in our 'world' database. The number of samples with DHA percentages above an arbitrary chosen cut off value at 0.60 mol% in the 'world' database amounted to 18/454 (4.0%), as opposed to 22/29 (75.9%) for Doromoni and 0/26 (0%) for Mwanga. The 'world' samples with DHA>0.60 mol% derived from Dominica (n=5; 29.4% of all samples), Antigua (n=3; 13.0%), St. Lucia (n=3; 25.0%), Netherlands (n=3; 1.3%), St. Vincent (n=2; 6.7%), Curaçao (n=1; 2.1%) and Surinam (n=1; 5.0%). The number of samples with AA>0.60 mol% amounted to 38/454 (8.4%) for 'world', our 10/26 (38.5%) for Mwanga, and 22/29 (75.9%) for Doromoni. Samples from the 'world' with AA>0.60% were from Curaçao (n=18; 38.3%), Jerusalem (n=5; 17.9%), St. Vincent (n=3; 10.0%), Antigua (n=3; 13.0%), St. Lucia (n=2; 16.7%), Palestine (n=2; 5.7%), Belize (n=2; 20.0%), Surinam (n=2; 10.0%), the Netherlands (n=1; 0.5%), and Dominica (n=5; 29.4%). There was a similar discrepancy between the relations of EPA and AA in Doromoni and the 'world' (Figure 1, bottom). Sixteen mothers (55.2%) from Doromoni had milk EPA and AA contents above 0.13 and 0.63 mol%, respectively, as compared to none of the mothers in our 'world' database.
Figure 3 shows the AA, EPA and DHA contents (top) and ratios (bottom) in fish from the Caribbean Sea, North Sea and Tanzanian freshwater lakes. Tanzanian lake fish had relatively high AA contents (Tanzania>North Sea; p=0.036) and high AA/DHA ratios (AA/DHA: Tanzania>Caribbean=North Sea;

Table 2. Doromoni and Mwanga milk FA composition compared with historical Dutch and Caribbean milk samples

	Doromoni (n = 29) median(min-max)	Mwanga (n = 26) median(min-max)	Dutch (n = 222) median(min-max)	Caribbean (n = 159) median(min-max)
6:0	0.31 (0.21-0.40)[a]	0.35 (0.19-0.60)	0.30 (0.17-0.52)[a]	0.17 (0.03-0.48)
8:0	0.58 (0.28-0.74)[a]	0.53 (0.32-0.97)[a]	0.66 (0.45-0.94)	0.67 (0.24-1.76)
10:0	3.06 (1.43-4.16)[a]	2.72 (1.47-5.35)[a]	2.73 (1.58-4.27)[a]	3.62 (0.57-6.15)
12:0	13.2 (4.37-20.4)[a,b]	10.9 (6.61-21.9)[a]	8.20 (2.91-15.7)	13.8 (4.12-34.9)[b]
14:0	12.9 (4.31-25.1)[a]	11.4 (8.23-21.9)[a]	7.89 (3.68-14.2)	11.5 (4.09-26.0)[a]
16:0	20.5 (15.0-26.4)[a]	22.6 (13.4-28.4)[a,b]	23.2 (14.5-28.8)[b]	20.9 (14.3-29.2)[a]
18:0	4.00 (1.87-6.45)[a]	4.08 (1.75-9.01)[a]	7.18 (4.84-9.68)	5.45 (2.14-8.77)
20:0	0.13 (0.05-0.52)[a]	0.13 (0.05-0.29)[a]	0.21 (0.03-0.37)	0.20 (0.07-0.91)
22:0	0.08 (0.04-0.33)[a,b]	0.07 (0.04-0.11)[a]	0.10 (0.05-0.21)	0.09 (0.00-0.34)[b]
24:0	0.09 (0.04-0.19)	0.07 (0.04-0.10)[a]	0.07 (0.03-0.16)[a]	0.07 (0.00-0.31)[a]
MCSAFA	30.4 (10.7-50.3)[a]	25.6 (17.3-46.7)[a]	19.9 (9.19-35.2)	30.6 (9.90-67.9)[a]
LCSAFA	24.9 (20.7-32.5)[a]	27.2 (15.5-37.9)[a]	30.8 (20.5-37.5)	26.8 (17.5-35.5)[a]
SAFA	55.6 (40.8-73.4)[a]	56.1 (41.8-66.9)[a]	51.0 (39.3-63.4)	57.5 (37.8-85.4)[a]
18:3ω3	1.21 (0.33-2.02)[a]	0.73 (0.26-1.80)[b]	1.02 (0.64-2.71)[a]	0.67 (0.27-2.00)[b]
20:5ω3	0.17 (0.05-0.35)	0.05 (0.02-0.11)[a]	0.05 (0.00-0.29)[a]	0.05 (0.00-0.36)[a]
22:5ω3	0.36 (0.13-0.65)	0.12 (0.04-0.25)[a]	0.12 (0.08-0.24)[a]	0.13 (0.00-0.31)[a]
22:6ω3	0.75 (0.25-1.47)	0.13 (0.04-0.35)	0.19 (0.09-0.84)	0.33 (0.09-1.63)
LCPω3	1.66 (0.53-2.83)	0.45 (0.22-0.67)[a]	0.36 (0.20-1.33)	0.52 (0.16-1.68)[a]
ω3	2.92 (0.92-4.73)	1.12 (0.49-2.43)[a]	1.42 (0.90-3.08)	0.98 (0.16-2.79)[a]
14:1ω5	0.08 (0.04-0.17)	0.18 (0.05-0.70)[a]	0.37 (0.03-0.69)	0.23 (0.05-0.52)[a]
18:2ω6	16.1 (8.95-23.2)[a]	15.4 (7.18-28.4)[a,b]	12.8 (6.01-28.2)[b]	11.3 (3.51-25.9)
18:3ω6	0.14 (0.09-0.33)[a]	0.11 (0.04-0.26)[a,b]	0.09 (0.03-0.20)[b]	0.07 (0.00-0.23)
20:2ω6	0.37 (0.21-0.44)[a]	0.35 (0.17-0.63)[a,b]	0.31 (0.17-0.57)[b]	0.32 (0.08-0.99)[a]
20:3ω6	0.52 (0.29-0.93)[a]	0.46 (0.26-0.78)[a]	0.33 (0.18-0.78)	0.38 (0.20-0.68)
20:4ω6	0.70 (0.50-0.93)	0.52 (0.27-0.79)[a]	0.37 (0.21-0.62)	0.50 (0.19-0.99)[a]
22:4ω6	0.16 (0.09-0.27)	0.12 (0.06-0.21)[a]	0.07 (0.04-0.16)	0.12 (0.00-0.50)[a]
22:5ω6	0.10 (0.02-0.17)	0.06 (0.02-0.10)[a]	0.03 (0.00-0.08)	0.05 (0.00-0.18)[a]
LCPω6	1.60 (0.96-2.26)	1.27 (0.64-1.90)[a]	1.11 (0.71-1.72)[a]	1.40 (0.59-3.25)[a]
ω6	17.5 (10.8-24.7)[a]	17.0 (8.04-30.5)[a,b]	14.0 (7.22-29.5)[b]	12.8 (4.14-27.4)
16:1ω7	1.39 (0.87-2.46)[a]	1.43 (0.66-2.61)[a]	2.33 (0.76-4.99)	2.58 (0.89-5.89)
18:1ω7	2.47 (1.26-5.98)[a]	2.48 (0.91-4.27)[a]	3.13 (1.57-5.34)	2.98 (0.79-7.63)
ω7	3.87 (2.26-8.44)[a]	3.89 (1.69-6.82)[a]	5.50 (2.66-9.40)	5.55 (1.96-10.3)
18:1ω9	17.8 (8.66-30.8)	22.4 (12.3-26.9)[a]	26.5 (19.1-34.5)	21.4 (7.17-34.6)[a]
20:1ω9	0.19 (0.08-0.47)[a]	0.23 (0.10-0.33)[a]	0.37 (0.22-0.69)	0.38 (0.06-1.10)
20:3ω9	0.05 (0.02-0.11)[a]	0.06 (0.03-0.11)[a]	0.05 (0.00-0.09)	0.06 (0.00-0.20)[a]
24:1ω9	0.05 (0.03-0.08)[a]	0.05 (0.03-0.08)[a]	0.04 (0.00-0.46)[a]	0.05 (0.00-0.27)[a]
ω9	18.2 (8.86-31.4)	22.8 (12.6-27.5)[a]	27.0 (19.5-35.1)	21.8 (7.28-35.6)[a]
MUFA	22.4 (12.5-38.9)[a]	27.0 (14.4-31.9)[a,b]	33.0 (22.2-44.7)	28.1 (9.54-42.9)[b]
PUFA	20.2 (13.3-26.5)[a]	18.6 (9.13-31.4)[a,b]	15.5 (8.41-32.2)[b]	13.9 (5.03-27.8)
LCPω3+LCPω6	3.15 (1.45-4.65)	1.64 (1.01-2.44)[a]	1.50 (1.05-2.21)[a]	1.92 (1.09-3.96)
ω3/ω6	0.15 (0.04-0.35)	0.08 (0.03-0.17)[a]	0.10 (0.04-0.28)	0.09 (0.01-0.27)[a]
LCPω3/LCPω6	1.09 (0.33-1.50)	0.38 (0.17-0.64)[a]	0.31 (0.17-1.50)[a]	0.35 (0.10-2.48)[a]
ALA/LA	0.08 (0.01-0.18)[a,b]	0.05 (0.02-0.15)[a]	0.08 (0.03-0.21)[b]	0.07 (0.03-0.11)[b]
AA/DHA	0.91 (0.55-2.61)	3.69 (1.70-9.29)	2.04 (0.30-3.75)	1.60 (0.15-3.83)
EPA/DHA	1.93 (0.48-4.11)	0.28 (0.08-1.10)[a]	0.25 (0.00-0.54)[a]	0.14 (0.00-0.50)

Data are in mol%. Statistics: data with a similar symbol do NOT differ, $p<0.01$. Note that NO comparison was performed between Dutch and Caribbean milk samples.

Abbreviations: FA, fatty acids; MCSAFA, medium chain saturated FA (sum of 6:0 up to 14:0); LCSAFA, long chain SAFA (sum with C>16); SAFA, saturated FA; LCPω3 and LCPω6, long chain polyunsaturated FA (C>20) of the ω3 and ω6 series; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; LA, linoleic acid (18:2ω6); ALA, α-linolenic acid (18:3ω3); AA, arachidonic acid (20:4ω6); DHA, docosahexaenoic acid (22:6ω3) and EPA, eicosapentaenoic acid (20:5ω3).

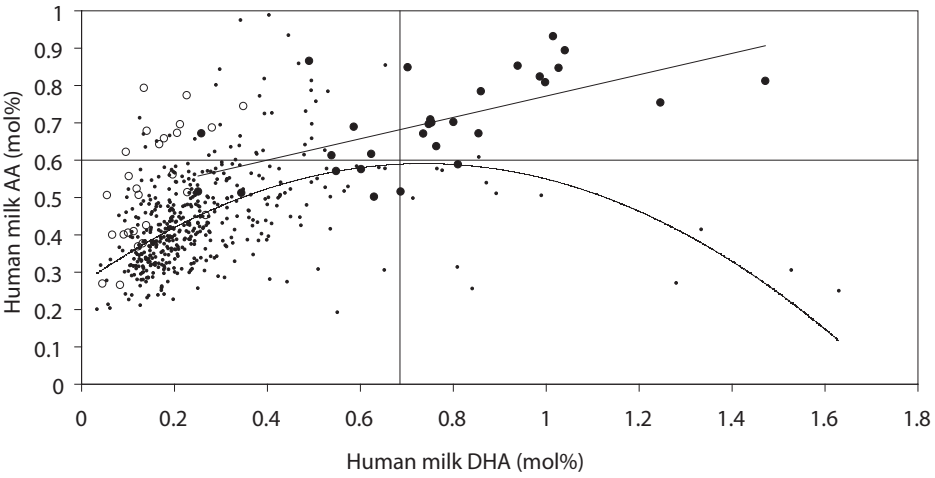

Figure 1. Relation between DHA and AA in human milk from Doromoni and Mwanga, as compared with data from or 'world' database. ●, Doromoni (n=29); ○, Mwanga (n=26); ●, World (n=454). AA, arachidonic acid; DHA, docosahexaenoic acid. Solid straight lines represent relation between data from Doromoni; the second grade polynomials are for data from the 'world' database. Samples from the 'world' derived from: Antigua (n=23), Belize (n=10), Curaçao (n=47), Dominica (n=17), Jerusalem (n=28), the Netherlands (n=222), Pakistan (n=10), Palestine (n=35), St. Lucia (n=12), St. Vincent (n=30) and Surinam (n=20). Horizontal and vertical lines represent arbitrary boundaries (see text) at DHA=0.65 and AA=0.61 mol%. For Doromoni the percentage samples with contents above these values were: 58.6%. Note the linear relationship between DHA and AA in Doromoni and the drop of AA at higher DHA contents in data from the 'world'.

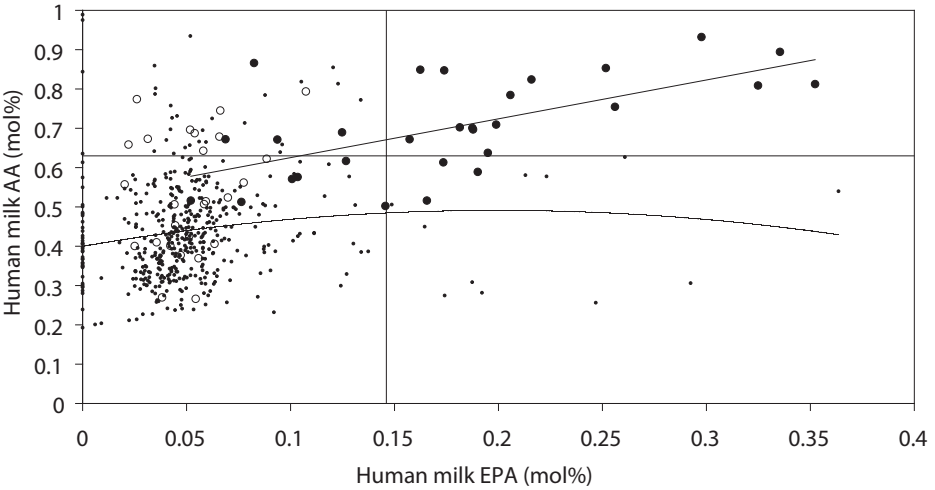

Figure 2. Relation between EPA and AA in human milk from Doromoni and Mwanga, as compared with data from or 'world' database. ●, Doromoni (n=29); ○, Mwanga (n=26); ●, World (n=454). AA arachidonic acid; EPA eicosapentaenoic acid. Solid straight lines represent relation between data from Doromoni; the second grade polynomials are for data from the 'world' database. For 'world' samples, see legend to figure 1. Horizontal and vertical lines represent arbitrary boundaries (see text) at EPA=0.13 and AA=0.63 mol%. For Doromoni the percentage samples with contents above these values was 55.2%. Note the linear relationship between EPA and AA in Doromoni and the drop of AA at higher EPA contents in data from the 'world'.

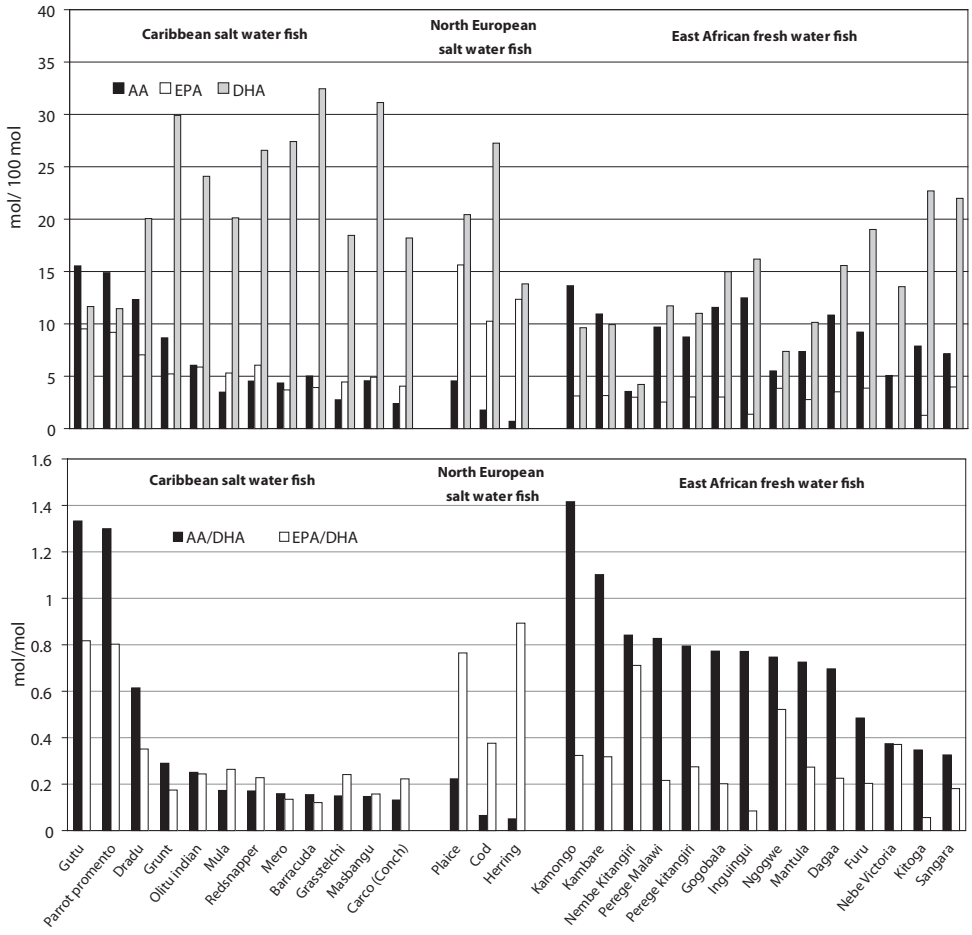

Figure 3. AA, EPA and DHA contents (top), and AA/DHA and EPA/DHA ratios (bottom) in fish from the Caribbean Sea, North Sea and Tanzanian freshwater lakes. Top: AA, arachidonic acid (closed bars); EPA eicosapentaenoic acid (open bars); DHA, docosahexaenoic acid (grey bars). Bottom: AA/DHA (closed bars); EPA/DHA (open bars). Gutu and Parrot promentu are parrot fish that live from coral, and grunt is a reef fish. These fish are not abundantly eaten in the Caribbean. Carco is the local name for a conch. Note the relatively high AA contents and AA/DHA ratio of the Tanzanian lake fish, the relatively high EPA content and EPA/DHA ratio of the North Sea fish, and the relatively high DHA content of the Caribbean fish.

p<0.015). The EPA content of the North Sea fish (North Sea>Caribbean>Tanzania; p<0.027) and its EPA/DHA ratio (North Sea>Tanzania; p<0.036) were relatively high, whereas the Caribbean fish had relatively high DHA contents (Caribbean>Tanzania; p=0.009). The AA/EPA ratio of the Tanzanian fish (mean 3.38; range 1.01-9.12) was higher than that of the Caribbean fish (1.14; 0.59-1.75, p<0.027)

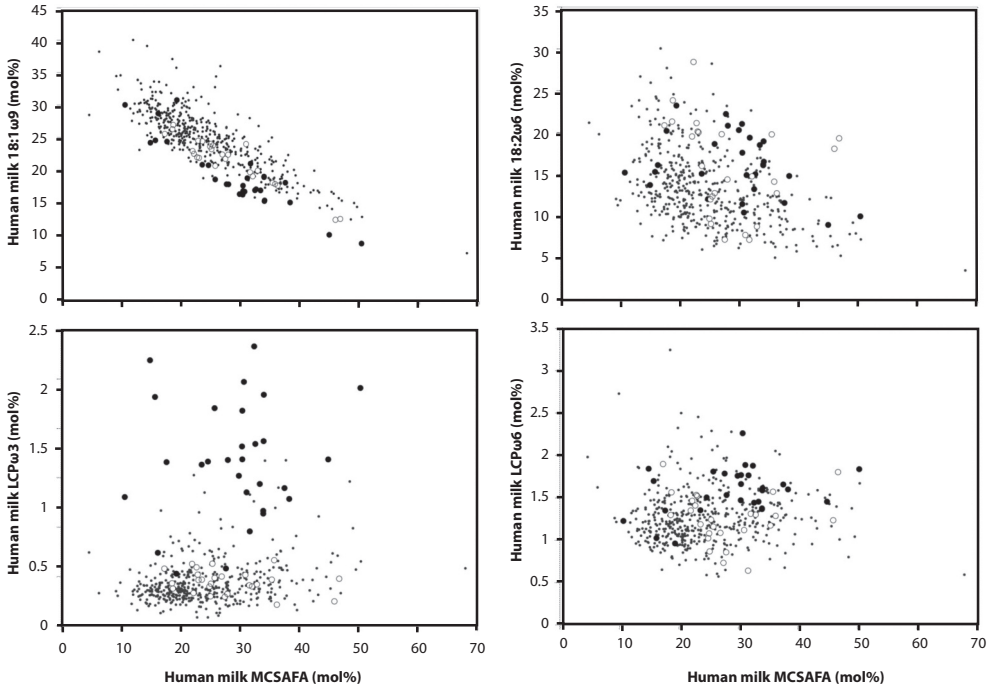

Figure 4. Relation between milk MCSAFA and 18:1ω9, 18:2ω6, LCPω3 and LCPω6, for data from the 'world' database, Doromoni and Mwanga. ●, Doromoni (n=29); ○, Mwanga (n=26); ●, World (n=454). MCSAFA, medium chain fatty acids (sum of 6:0 up to 14:0); 18:1ω9, oleic acid; 18:2ω6, linoleic acid; LCPω3 and LCPω6, long chain polyunsaturated fatty acids (C≥20) of the ω3 and ω6 series, respectively. For results of multilinear regression analyses see Table 3. Note that MCSAFA compete with 18:1ω9 and 18:2ω6, but not to an appreciable extent with LCPω3 and LCPω6.

and was much higher than that of the North Sea fish (0.17; 0.06-0.29, $p<0.009$) (not shown in Figure 2). Figure 3 and **Table 3** show the relations between the milk MCSAFA content on the one hand and 18:1ω9, 18:2ω6, LCPω3 and LCPω6 on the other for data from 'world' database, Doromoni and Mwanga.,

The inverse relations between MCSAFA, and 18:1ω9 and 18:2ω6 exhibited similar slopes for all groups. However, inspection of the intercepts revealed that Doromoni and Mwanga had lower 18:1ω9 (Doromoni<Mwanga) and higher 18:2ω6 (Doromoni=Mwanga) at all MCSAFA contents, compared with the 'world'. Although there was a positive relation between MCSAFA and LCPω3MCSAFA had negligible effect on LCPω3 (see **Figure 4** and slope in Table 3). MCSAFA proved unrelated to LCPω6. Doromoni milk had higher LCPω3 and LCPω6 at all MCSAFA contents, compared to both Mwanga and the 'world'.

The fatty acid composition (in mol%) of the local conflower oil was: 14:0 (0.33); 16:0 (8.05); 18:0 (4.40); 22:0 (1.35); 18:3ω3 (0.33); 18:2ω6 (52.30); 16:1ω7 (0.63); 18:1ω7 (1.24); and 18:1ω9 (31.37).

Table 3. Relations of MCSAFA with 18:1w9, 18:2w6, LCPw3 and LCPw6, as obtained from multiple linear regression analyses

y-variable	Explained variance (%)		World	Doromoni	Mwanga
Ln(18:1w9)	79	Slope	- 0.022	- 0.022	- 0.022
		Intercept	3.724	3.541	3.675
Ln(18:2w6)	24	Slope	- 0.018	- 0.018	- 0.018
		Intercept	2.976	3.275 [a]	3.195 [a]
Ln(LCPw3)	38	Slope	0.010	0.010	0.010
		Intercept	- 1.174 [a]	0.141	- 1.174 [a]
Ln(LCPw6)	5	Slope	0	0	0
		Intercept	0.195 [a]	0.432	0.195 [a]

Best linear models were obtained after Ln-transformation of the y-variables (i.e. 18:1ω9, 18:2ω6, LCPω3 and LCPω6)
All slopes are significantly different from zero at $p<0.001$, except for MCSAFA-Ln(LCPω6) relationships, similarly $p<0.001$ for all between-group differences of intercepts, except for those indicated with ([a])

DISCUSSION

In search of the milk AA and DHA contents of our African ancestors we investigated the milk of 29 lactating women living in Doromoni near lake Kitangiri (Tanzania). These women consume sunflower oil-fried local fish as the only animal lipid source, together with maize and local vegetables. The dietary macronutrient composition of the Doromoni women is not comparable with what is nowadays assumed to be our ancient diet. Based on the study of hunter-gatherers, O'Keefe et al.[14] estimated that the diet of *homo sapiens* up to 10,000 years ago was high in protein (19-35 energy%), moderate in carbohydrates (22-40%) and moderate in fat (28-47%). The fat fraction was composed of moderate saturated fat, high monounsaturated fat and moderate polyunsaturated fat, and provided a high ω3 fatty acid intake from α-linolenic acid and lacustrine- or marine-based foods (EPA and DHA). In contrast to this composition, the diet of the Doromoni women is rich in carbohydrates and linoleic acid from sunflower oil, as became apparent from the interviews and their milk fatty acid composition (Table 2). High carbohydrate intakes increase the milk MCSAFA content.[25-27] The linoleic acid content of the Doromoni milk proved even higher than the linoleic acid content in Western countries (such as the Netherlands) with typically abundant consumption of polyunsaturated fats from vegetable oils. Based on fatty acid interrelationships (Figure 4**,** Table 3) and literature data, we nevertheless consider the LCP content of the Doromoni milk close to that of the diet of early *homo sapiens*. The *de novo* synthesized MCSAFA from glucose in the lactating breast do not compete with LCP for incorporation into milk lipids, but rather with oleic acid and others like linoleic acid (Figure 3). This is in line with the observation that a high carbohydrate intake increases the milk MCSAFA content at the expense of oleic, stearic and to lesser extent palmitic acids.[25,26] In addition, milk linoleate and AA do not correlate,[31] and increases of the milk EPA and DHA do not

occur at the expense of linoleic acid.[32,33] The 'relative resistance' of milk LCP towards exchange with MCSAFA is unlikely to be due to the preferential location of LCP in the milk phospholipid fraction and MCSAFA in the triglyceride fraction.[31] MCSAFA in milk from mothers with carbohydrate-rich diets incorporate equally in phospholipids and triglycerides, and depress both the PUFA and LCP contents of the phospholipid fraction.[34] Reduction of LCP in milk phospholipids by a carbohydrate-rich diet might be important since LCP (at least AA) from dietary phospholipids are 2.1 fold more effective than LCP from triglycerides for accretion in neonatal brain.[35] On the other hand, the vast majority of the milk MCSAFA and LCP derive from triglycerides, which are on a weight bases 100 times more abundant than phospholipids.[31] Taken together, we suggest that the LCP content of the Doromoni milk may be close to that of early *homo sapiens*, because of the similarity of the abundant consumption of East-African lacustrine-based foods, the resulting life-time high intakes of both AA and DHA, and because the disparities in the Doromoni and ancient diets (i.e. different carbohydrate and linoleic acid intakes) are unlikely to have affected the human milk AA and DHA contents. It is, however, likely that the higher carbohydrate intake in Doromoni, as compared with the ancient diet, lowered the LCP content of the Doromoni milk phospholipid fraction to some extent.

The high contents of both AA and DHA in the milk of Doromoni women proved unprecedented when compared with the data of our 'world' human milk fatty acid database (Figure 1). The Doromoni milk AA content was higher and its DHA content lower than previously reported data on the milk of Malays and Indians living in Penang,[36] who were also noted to consume AA and DHA rich local fish. Their AA (0.70 vs 0.64 mol%) and DHA (0.75 vs 0.71 mol%) were however comparable with those of Chinese women living in Penang, who, next to vegetable oil-fried fish, consume a variety of, AA-rich,[37] meat products, including chicken, beef and pork. Compared with Inuit women, those in Doromoni had considerably lower milk DHA (0.75 vs 1.4%) and EPA (0.17 vs 1.1%), but somewhat higher AA (0.70 vs 0.6%).[38] The rather unique high Doromoni milk LCP contents in combination with its almost 1:1 AA/DHA ratio is without doubt due to the consumption of fish from the East-African freshwater lakes. These fish have high contents of both AA and DHA, with AA/DHA ratio's of about 0.75 in conjunction with relatively low EPA contents (Figure 3). Oppose this notion seems to be that no increase of milk AA could be detected in Palestinian women during short term AA supplementation (300 mg/day; one week).[22] It is on the other hand known that prolonged AA supplementation (1.7 g/day, 50 days) augments the AA contents of various lipid compartments, notably those of the plasma phospholipids, but also of erythrocytes, platelets and fasting plasma triglycerides, free fatty acids and cholesterol esters.[39,40] In agreement with this, higher whole plasma AA was found in a fish eating community living at the shore of lake Nyasa (Tanzania), as compared with a vegetarian community living 50 miles inland.[41] These subjects also had higher whole plasma EPA and DHA, and higher EPA/AA and DHA/AA ratios. Reaching high whole body LCP status comparable with these communities might be a prerequisite to increase the milk LCP content to the extent of Doromoni, since milk LCP derive mostly from body stores and to lesser extent directly from the diet. It was estimated that 90% of milk AA does not derive from direct intestinal absorption, while this amounts to 70-80% for

linoleic acid and DHA.[33,42] Taken together this implies that the higher milk AA content in Doromoni derives mainly from a life-long abundant AA intake from local fish and that this applies to DHA to somewhat lesser extent. It also implies that, given the large distribution volumes for body fatty acids (i.e. cellular phospholipids and adipose tissue triglyceride stores), Western mothers may require a sizeable combination of time and LCP dosage to reach milk AA and DHA contents comparable with those in Doromoni.

The AA/EPA ratio of the Tanzanian fresh water fish was higher than that of the Caribbean fish and much higher than that of the North Sea fish. This difference tracks down to the origination of the LCPω3 in salt water fish from EPA-rich marine phytoplankton and zooplankton, whereas most freshwater fish are capable of producing their own DHA from α-linolenic acid.[18] It is possible that the high AA contents and high AA/EPA ratio of the East-African lake fish prevented AA and EPA from becoming competitors for augmentation of AA body status, as suggested by comparison of the milk EPA-AA and DHA-AA relations in Doromoni and certain other countries with high EPA and DHA intakes from fish (Figure 1 and Figure 2). The latter countries were notably Caribbean islands, such as Dominica, Antigua, St. Lucia and St. Vincent, where fish is eaten with lower AA content but also lower AA/EPA ratio. In addition it has been reported that collaboration, rather than competition, between AA and EPA, and possibly also between AA and DHA, may occur in certain circumstances notably at low EPA and DHA dosages.[23,24] In line with this notion we have previously reported that short term supplementation with AA did not increase human milk AA, but that supplementation with AA, EPA and DHA tended to increase both AA and LCPω3 after 1 week.[23] This suggests that not only dietary AA and DHA intakes are determinants of their ultimate body status, but that the AA/EPA and possibly the AA/DHA ratio, might be of importance as well. Apart from this dietary 'LCP balance' it might also be important in which dietary lipid fraction(s) the AA, EPA and DHA are located. As previously noted, AA in dietary phospholipids was found to be a more efficient dietary source to augment the brain AA content of neonatal baboons, when compared with AA in dietary triglycerides.[34] Lean fish, like those from the East-African lakes and Caribbean Sea, are in this respect different from the fatty fish from the North Sea, in the sense that AA and DHA are both located in phospholipids of lean fish, and that in fatty fish AA is mainly located in phospholipids and DHA is located in both phospholipids and the abundant triglycerides. Consumption of fish with balanced AA/EPA/DHA composition and with AA and DHA located in phospholipids, i.e. East-African lake fish, may be the most efficient way to augment the AA and DHA body status.

The present results shed doubt on the validity to use milk from Western mothers as the gold standard for the addition of LCP to infant formulae. Although there is no solid evidence for long term adverse effects of feeding infant formulae without LCP, it is becoming increasingly clear that dietary LCP have many effects ranging from their nowadays well known influence on visual and neurological function to the more recently discovered and currently intensely studied influence on the expression of our genome.[10-13] The consequences of subclinical deficiencies are difficult to objectify, since they may operate over long periods that confound their identification as the

causative insults. For instance, a link between perinatal LCP status and obesity, hypertension, insulin resistance, and coronary heart disease has been suggested,[43] but how are we to get proof with hard endpoints according to the rules of evidence based medicine? As much as we criticize the LCPω3 content and the out of proportion linoleic acid/α-linolenic acid ratio of contemporarily Western diets, we might criticize the milk fatty acid composition of Western mothers. The DHA and EPA contents are the most variable human milk fatty acid components worldwide and the previously established low biological variation of its AA content[8] has in this study been refuted as deriving from a selection bias. It is virtually impossible and certainly unaffordable to perform randomized controlled trials with each of the components of our diet and preferably their combinations, which argues in favor of clues that should come from the diet on which our genome has evolved. The milk AA, DHA and EPA contents of the Doromoni women might provide us with a clue to optimize infant formulae and perhaps also the milk fatty acid composition of Western women.

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CHAPTER 3.2

Milk in the island of Chole (Tanzania) is high in lauric, myristic, arachidonic and docosahexaenoic acids, and low in linoleic acid

Reconstructed diet of infants born to our ancestors living in tropical coastal regions

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ABSTRACT

Background. We need information on the diet on which our genes evolved.

Objective. We studied the milk fatty acid (FA) composition of mothers living in the island of Chole (Tanzania, Indian Ocean). These mothers have high intakes of boiled marine fish and coconut, and consume plenty amounts of fruits and vegetables.

Design. The outcome was compared with 3 fish-eating tribes living along Tanzanian freshwater lakes (Kerewe, Nyakius, Nyiramba), 4 tribes living in the Tanzanian inland (Hadzabe, Maasai, Sonjo, Iraqw) and our milk FA database.

Results. Milk from Chole contained high levels of 12:0 (20.17 g%), 14:0 (21.19), 12:0/14:0 ratio (0.92 g/g), arachidonic acid (AA, 0.50 g%) and docosahexaenoic acid (DHA, 0.73), but low levels of linoleic acid (LA, 4.23). The combination of a high medium chain fatty acid (MCSAFA; <C16) content and 12:0/14:0 ratio derives notably from coconut consumption, as opposed to a carbohydrate rich diet, while non-existent use of vegetable oils explains low LA. Milk AA/DHA ratios of the 4 fish-eating groups were related to the AA/DHA ratios of the available fish. Chole MCSAFA and LA did not fulfill Western recommendations for formulae, while AA and DHA were well above minimum levels.

Conclusions. The Chole milk FA composition is likely to reflect the dietary FA composition of babies born to our ancient ancestors living in East-African coastal regions. The poor compliance with present recommendations raises questions regarding the validity of recommendations that are based on milk from Western mothers consuming diets that confer high risk of diseases typical for affluent countries.

INTRODUCTION

Evolutionary medicine acknowledges that our genes have been selected against the background of a certain environment, including diet, and that this environment consequently supports a state of optimal homeostatic control. We have since the agricultural revolution (i.e. some 10,000 years ago) gradually changed our diet and accelerated these changes from the beginning of the industrial revolution (i.e. 100-200 years ago).[44-47] These changes, together with a sedentary lifestyle have caused a mismatch with our slowly adapting genome. The resulting conflict between our genes and the current environment is at the basis of many, if not all, typically Western diseases, including coronary artery disease (CAD), type 2 diabetes mellitus, osteoporosis, acne vulgaris and certain cancers, including those of the prostate, breast and colon. Return to our ancient diet as translated to modern society is indicated, since it might increase our number of years in health, rather than increase our life expectancy.[48]

The dietary composition of our ancient ancestors may to some extent be reconstructed from archeological discoveries. The sites at which their fossil remains have been discovered are in support of the notion that evolution to *Homo sapiens* took place on an ω3-rich diet from East-African ecosystems that were notably located in places where the land meets with water.[16,49,50] Food from these ecosystems is rich in iodine, vitamins A and D, and ω3-fatty acids from both vegetable origin and fish. This composition seems somewhat abandoned since the Out-of-Africa Diaspora, since deficiencies of these particular nutrients are among the most widely encountered in the current world population.[16,50-52] Iodine is added to table salt in many countries, and margarines and milk have become popular food products for fortification with vitamins A and D. The dietary composition of our ancestors has also become clear from our (patho)physiology. Epidemiological data demonstrated a negative association of fish oil with CAD,[53,54] and of fish consumption with (postpartum) depression,[55,56] while landmark trials with alpha-linolenic acid (ALA) {de Lorgeril, 1999 205 /id} and fish oil[57] in CAD, and with eicosapentaenoic acid (EPA) in depression and schizophrenia[58] supported the causality of these relations.

We[28,59] recently criticized recommendations for the fatty acid composition of infant formulas as issued by various nutritional authorities[7,60-69]. These recommendations are based on human milk data from Western mothers who consume Western diets and who are consequently at high risk for typically Western diseases. For instance, there is no rationale in a recommendation of 8-35 g% linoleic acid (LA; 18:2ω6), when we take into account that LA consumption has sharply increased in the past decades because of augmented consumption of ω3-poor vegetable oils, there is no scientific evidence to restrict the medium chain saturated fatty acids (MCSAFA; <C16) 12:0 and 14:0 to ≤15 g%, and the criteria for the long chain polyunsaturated fatty acid (LCP; ≥20 carbon atoms and ≥3 methylene-interrupted cis-double bonds) arachidonic acid (AA)≥ 0.35 g% and docosahexaenoic acid (DHA)≥ 0.20 g% seem to neglect the declining intakes of LCPω3 and possibly AA. Tanzanian mothers with high consumption of freshwater fish as only source of animal protein and fat had both high AA and DHA contents in their milk, when compared with those of Western mothers and the

recommendations derived of these.[59] Above 15 g% 12:0 and 14:0 milk contents are, on the other hand, especially encountered in women with high intakes of carbohydrates from grains and corn. Carbohydrate-rich diets are not consistent with present notions on the macronutrient composition of our ancient diet[45] and it may consequently be questioned whether the resulting high MCSAFA contents in milk have been part of newborn diets in the past.

Also consumption of coconuts is known to increase milk MCSAFA, notably 12:0 and 14:0,[70] since coconut oil characteristically contains about 47 g% 12:0, 18 g% 14:0, 8% 8:0, 6% 10:0 and 1% 6:0.[71] Availability of coconuts is currently typical for most tropical seashores. Whether coconuts have been part of the diet of early *Homo sapiens* is uncertain, since no coconut fossils have been found in East Africa. On the other hand not many fossilized coconuts have been found anywhere, which is probably because eventually they tend to become fully recycled on the seashores of the hot and humid tropics.[72] One of the exceptions is a silicified coconut fruit from the Chinchilla sands in southern Queensland (Australia), which was dated to the late Pliocene, about 2 mya, suggesting an already wide distribution range at that time.[73] Coconuts are capable of developing after having floated in the sea for up to 110 days and their theoretical range of dissemination by floating is estimated to be anywhere between the African East coast and the American West coast, wherever currents are favourable.[74] It seems therefore plausible that coconuts have been available to early humans and that they may have served as easy meals and drinks for opportunistic hunter-gatherers[72] living along the African coast, while also the Out-of-Africa Diaspora is likely to have occurred along the shores of the Indian Ocean into Asia.[75,76]

In the present study we investigated the milk from mothers living in Chole, which is a small island in the Indian Ocean, close to the Tanzanian mainland, located South of the much larger Mafia Island. The inhabitants of Chole have high intakes of local marine fish and coconut, and consume plenty amounts of fruits and vegetables. In contrast to our former fish-eating study population[59] they do not use vegetable oils for cooking and have low intakes of carbohydrates from grains or corn, which is likely to cause much lower milk LA and *de novo* synthesized MCSAFA from glucose. Their consumption of coconut may be expected to increase milk 14:0, but especially 12:0. For comparison with the milk from Chole we carefully selected 3 populations or tribes living along Tanzanian freshwater lakes (i.e. Lakes Victoria, Malawi and Kitangiri) and 4 living in the Tanzanian inlands. For a better understanding of the milk LCP contents we also analyzed the fatty acid composition of various local freshwater and marine fishes.

SUBJECTS AND METHODS

Study design and study groups

Tanzania is inhabited by about 126 different tribes.[77] Intermarriage is rare and, dependent on the directly surrounding environmental circumstances, many of the tribes have remained unique with respect to their diets. Our primary interest was the people living in the island of Chole. In addition we recruited 3 groups of fish-eating controls (Kerewe, Nyakius, Nyiramba), 4 groups from the inlands

(Hadzabe, Maasai, Sonjo, Iraqw) and also compared with historical milk fatty acid data from Dar-es-Salaam.[8] The latter group was composed of lactating mothers who attended the local University maternity ward of the Muhimbili Medical Center in Dar-es-Salaam in 1970. The milk samples were collected by one of us (ERB) who at that time worked in the University Hospital of Dar-es-Salaam as a pediatrician. The Chole population is composed of a mixture of people from the African inlands, who were transported and concentrated in the coastal areas by Arab slave traders in the 19th century. The staple foods are coconut and preferably boiled marine fish, which are combined with free fruits (oranges, mangos and bananas) growing all over the island and an occasional flying fox, of which one of the last colonies in the world resides in this island. Fish-eating controls were recruited from the people of Ukerewe (the Kerewe), the Nyakius people and the Nyiramba people. Ukerewe is an island located in the South-East of Lake Victoria. The island is abundant in fruits (mandarin, orange, mango and several species of bananas) and the Kerewe diet is rich in, preferably boiled, freshwater lake fish. The carbohydrate-rich porridge 'ugali' (local name for corn wheat porridge) is consumed as part of their daily diet. The Nyakius people are members of a tribe living on the northern shores of Lake Malawi (also named lake Nyasa or Nyassa) around Matema Beach. The area is extraordinarily fertile and provides plenty of fruits (e.g. mango, orange, banana), as well as abundant freshwater fish from the lake. Corn wheat is to a lesser extent part of their diet, while cassava ('muhogo', in Kiswahili) is their major carbohydrate source. The Nyiramba people live around a freshwater lake in the village of Doromoni, which is located at the shores of Lake Kitangiri, 100 km south-west of the alkaline lake Eyasi. Their diet consists of freshwater fish that is mostly fried in local corn oil (52 g% LA; 31 g% oleic acid). Vegetables and especially fruits are scarce and difficult to obtain, and therefore not part of the daily dietary intake. The fatty acid composition of the milk from the Nyiramba people has previously been described[59] but is incorporated to facilitate comparison.

Controls living in the Tanzanian inlands were recruited from the Hadzabe, the Maasai, the Sonjo and the Iraqw people. The Hadzabe are among the last hunter-gathering tribes in the world. Largely driven out of their original territory into the arid areas, unattractive for crop cultivation, they nowadays depend at least partially on food distribution (corn flour) from a local Lutheran Hospital. Hunting is still daily practice, while roots, honey and berries are gathered. Corn and corn oil have inevitably become a large part of their contemporary diet. The second group is the Maasai. After having been forced out of the Serengeti National Park they still live relatively traditional lives in the Conservation Area of Ngorongoro since 1958, where they are the only ones allowed living and herding their stock. They are pastoral people and the diet of those not involved in daily (eco) tourism consists of curdled milk and occasional meat that has recently become replenished with some ugali (corn porridge). In between the Maasai, in a heavily fortified village located in the only green valley in miles, live the Sonjo. They combine hunting with agriculture and in their palisade-surrounded valley they have to their disposal a wide variety of fruits. Small local markets contribute to the trade of milk and fruits between the Maasai and Sonjo women. Finally we recruited from the Iraqw people, who reside in the region south of Lake Eyasi, where they live as farmers in villages within the Mbulu

district. Subjects were recruited in Mwanga, a small village in the dry highlands between Kiomboi and Mbulu, close to the Haidom Lutheran Hospital. Most of the mothers are well nourished. Due to poor herding grounds and consequently small herds of cattle that are kept for the production of milk, notably the women should be regarded as vegetarians. Fish is not eaten because of the traditional beliefs that it is a snake-like creature and therefore not edible. The fatty acid composition of the milk from the Iraqw people has previously been described (59) but again shown to facilitate comparison.

Samples and analyses

About 5 mL milk samples were collected by one of us or by one of the local medical doctors or nurses in local dispensaries. Women were eligible to participate if they gave informed consent, were breastfeeding, apparently healthy and well nourished, and had delivered an apparent healthy child more than 10 days prior to their visit to the local dispensary. Anthropometric data were recorded and are presented in **Table 1**. Data on age, parity, dietary habits and duration of lactation were obtained from the medical records or by interviews in Kiswahili as conducted by one of us (RSK). Fish were caught by line, purchased from local fisherman or bought at local markets at Lake Victoria, Lake Malawi and in Dar-es-Salaam by one of us (RSK). Small (1 cm^3) portions of edible meat were taken from just above the backbone, in front of the dorsal fin. The study was approved by the National Institute for Medical Research in Dar-es-Salaam (NIMR/HQ/R.8a/Vol. IX/145, dated June 16, 2003) and was in agreement with the Helsinki declaration of 1975 as revised in 2000.

All samples were immediately processed after collection. Following gentle mixing an aliquot of 100 μL of milk was pipetted into a teflon-sealable tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and internal standard (50 μg 17:0 in 100 μL methanol). In this ready-to-transmethylate mixture fatty acids are stable for months at room temperature and in the dark. Samples of fish were stored in the same methanol-HCl mixture. All samples were transported at room temperature to the University Medical Center Groningen (The Netherlands) for fatty acid analysis.

Analyses of fatty acid methyl esters (FAME) in milk and fish were performed by capillary gas chromatography/flame ionization detection according to previously described procedures[78] Briefly, milk samples were fortified with a series of odd-carbon chain numbered fatty acid internal quantification standards (5:0 up to 17:0). Transmethylation of both milk and fish occurred at 90 °C for 4 h and FAME were subsequently extracted into hexane. Long-chain fatty acids (≥16:0) were quantified on the basis of the added 17:0. Medium chain fatty acids (6:0 up to 14:0) were quantified with use of 5:0 - 15:0 as internal quantification standards.[30] Fatty acid compositions and their ratios were expressed in g%, mol%, g/g and mol/mol.

Data evaluation

Statistical analyses were performed with SPSS version 12.0.1 (SPSS Inc, Chicago, IL). Correlations

Table 1. Anthropometrics of Tanzanian mothers and their children

	Chole (n=20)	Kerewe (n=30)	Nyakius (n=30)	Nyiramba (n=35)	Hadzabe (n=28)	Maasai (n=27)	Sonjo (n=9)	Iraqw (n=18)
Mother								
Maternal age (years)	26 (15-40)	27 (15-38)	24 (16-37)	24 (16-36)	27 (18-43)	24 (17-38)	29 (21-36)	25 (18-42)
Parity	3 (1-7)	3 (1-9)	2 (1-5)	2 (1-6)	4 (1-8)	3 (1-8)	5 (2-8)	3 (1-7)
Maternal length (cm)	157 (145-171)	159 (145-170)	nm nm	157 (143-172)	152 (141-159)	159 (151-170)	162 (152-173)	158 (143-167)
Maternal weight (kg)	52 (40-73)	50 (38-65)	nm nm	56 (40-70)	50 (42-55)	54 (41-75)	58 (42-71)	56 (46-74)
Maternal BMI (kg/m2)	21 (17-28)	20 (16-26)	nm nm	23 (18-30)	22 (18-25)	21 (16-27)	22 (18-28)	22 (18-29)
BMI<18.5 (%)	25	33	nm	3	7	4	11	11
BMI 18.5-24.9 (%)	65	60	nm	80	93	89	67	67
BMI 25.0-29.9 (%)	10	7	nm	14	0	7	22	22
BMI>30 (%)	0	0	nm	3	0	0	0	0
Infant								
Child age (days)	253 (15-589)	259 (65-495)	285 (37-630)	128 (13-405)	291 (30-750)	176 (46-454)	174 (12-373)	108 (23-342)
Child gender (% male)	75	43	53	60	67	56	44	50
Child length (cm)	65 (46-84)	64 (54-77)	67 (54-82)	60 (47-73)	67 (51-89)	59 (47-72)	59 (44-75)	59 (52-66)
Child weight (kg)	8.2 (2.5-12.5)	7.6 (5.0-10.5)	8.4 (2.9-12.8)	6.2 (3.5-9.8)	9.7 (6.0-16.0)	7.1 (4.8-11.2)	7.0 (4.0-9.8)	5.9 (4.5-8.0)
Child HC (cm)	43 (34-50)	44 (40-49)	45 (38-49)	40 (34-47)	45 (38-49)	43 (39-49)	43 (36-49)	41 (38-45)

Data are mean (range); nm, not measured. Maternal data refer to postpregnancy length, weight and body mass index (BMI), HC= head circumference.

were studied with the aid of the Pearson correlation test. Between-group differences were tested by Mann Whitney U-test at $p<0.05$. Correction was made for type-1 errors. Most of the differences were however evaluated by visual comparison with the aid of box plots, since it was rather our intention to point at differences in trends between e.g. Tanzanian data and those of other countries, or between fish-eating and inland-living tribes within Tanzania. A multitude of between-group comparisons was not considered to add to our line of reasoning. For comparison with other countries we used the 'world data base' on human milk fatty acid compositions that we have collected in the past 25 years, using the same milk sampling techniques and analytical methods. Our present 'world data base' consists of people from Antigua (AG); Curaçao-Netherlands Antilles (NA); Belize (BZ); Dominica (DM); Jerusalem-Israel (IL); St. Lucia (LC); The Netherlands (NL); Pakistan-Islamabad (PK); Jerusalem-Palestine (PS); Surinam (SR); St. Vincent (VC) and the previously mentioned historical milk fatty acid data from Dar-es-Salaam (TZ-DAR). The milk fatty acid compositions in Tanzania were also evaluated in the context of current recommendations for infant formulae fatty acid compositions, as issued by various organization (7,60-69). The outcomes were compared with the corresponding data as derived from our 'world data base'.[8,28]

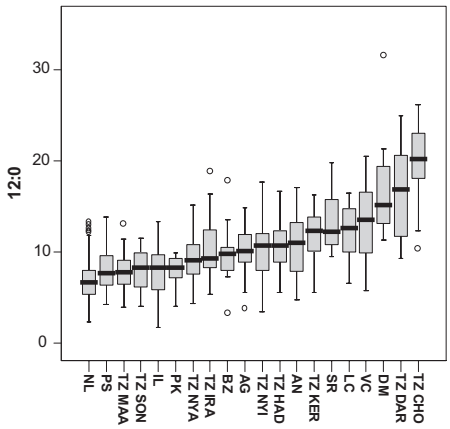
RESULTS

Relatively high numbers of women in Chole (25%) and Kerewe (33%) had low BMI according to Western standards (Table 1, BMI <18.5 kg/m^2). Between group analyses of BMI <18.5 and BMI ≥18.5 kg/m^2 for Chole showed a significant difference for the milk 12:0/14:0 ratio ($p=0.047$), with higher values for subjects with high BMIs. For Chole, negative relationships were observed between BMI and 14:0 ($r=-0.542$; $p=0.040$), BMI and 24:0 ($r=-0.473$; $p=0.030$), and a positive relation between BMI and the 12:0/14:0 ratio ($r=0.601$; $p=0.004$). For the Kerewe, between group analyses between BMI <18.5 and BMI ≥18.5 kg/m^2 gave significant differences with higher 18:0 ($p=0.037$), LA ($p=0.009$), ω6 ($p=0.009$) and PUFA ($p=0.017$) for subjects with high BMI. The Kerewe showed positive relationships between BMI on the one hand and 20:0 ($r=0.389$; $p=0.037$), LA ($r=0.407$; $p=0.028$), 20:2ω6 ($r=0.439$; $p=0.017$), 20:3ω6 ($r=0.513$; $p=0.004$), ω6 ($r=0.416$; $p=0.025$) and PUFA ($r=0.369$; $p=0.049$), on the other hand. The results of the fatty acid analyses (in g% and g/g) for milk samples from Chole, the Kerewe, the Nyakius, the Nyiramba, the Hadzabe, the Maasai, the Sonjo and the Iraqw people, together with the historical data from Dar-es-Salaam, are presented in **Table 2**. All data have been obtained by using a single analytical method {Steege van der, 1987 46 /id} Comparisons of Tanzanian milk fatty acid contents with those of our 'world data base' are presented as box plots for 12:0, 14:0, 16:0, LA, DHA and AA in **Figure 1. Table 3** shows the percentages of milk samples from the various groups in Tanzania that do *not* fulfill the recommendations for 12:0, 14:0, LA, 18:3ω3, AA, EPA, DHA, LCPω3 and LCPω6 in infant formulae. Milk of Chole mothers showed high contents of 12:0 (Table 2, 20.17 g%), compared with all other groups. These levels were only approximated by those of Dar-es-Salaam (16.89 g%) and the Kerewe (12.28 g%). Chole and Dar-es-Salaam are both located in the Tanzanian coastal region, where palm trees are abundant and coconuts freely available. High levels

Table 2. Milk fatty acid composition and ratios of the Tanzanian study groups

	Chole (n=20)	Kerewe (n=30)	Nyakius (n=30)	Nyiramba (n=35)	Hadzabe (n=28)	Maasai (n=27)	Sonjo (n=9)	Iraqw (n=18)	Dar es Salaam (n=11)
8:0	0.45 (0.31-0.60)	0.33 (0.15-0.54)	0.27 (0.18-0.43)	0.33 (0.17-0.48)	0.38 (0.17-0.62)	0.31 (0.22-0.42)	0.29 (0.21-0.37)	0.33 (0.19-0.61)	0.55 (0.40-0.80)
10:0	2.27 (1.67-3.44)	1.82 (0.76-2.79)	1.70 (1.04-2.81)	2.09 (0.99-3.04)	2.26 (0.89-3.58)	1.94 (1.34-2.75)	1.69 (1.00-2.20)	2.06 (1.03-4.00)	2.79 (1.56-3.62)
12:0	20.2 (10.4-26.1)	12.3 (5.58-16.3)	9.06 (4.35-15.1)	10.7 (3.49-17.6)	10.7 (5.60-16.6)	7.82 (3.90-13.1)	8.28 (4.01-11.5)	9.26 (5.33-18.8)	16.9 (9.29-24.9)
14:0	21.2 (11.2-31.1)	20.3 (13.2-28.8)	12.5 (4.89-25.8)	11.4 (3.89-24.6)	14.2 (6.60-18.6)	9.22 (5.05-16.2)	10.9 (6.26-13.0)	11.2 (7.53-21.1)	12.9 (7.21-27.4)
16:0	24.9 (20.6-28.7)	25.7 (22.9-32.3)	25.9 (17.9-32.8)	20.4 (14.5-26.0)	23.2 (15.5-30.3)	27.9 (23.2-34.1)	26.0 (22.2-32.0)	22.3 (13.8-27.7)	19.1 (14.9-28.7)
18:0	3.64 (1.51-4.67)	4.83 (3.53-5.95)	4.74 (2.07-8.40)	4.42 (2.15-6.90)	3.79 (3.10-7.06)	6.04 (3.81-12.6)	6.11 (4.26-15.8)	4.50 (1.99-9.23)	4.22 (1.26-4.88)
SAFA	75.3 (57.2-82.6)	67.1 (53.3-78.3)	56.4 (44.5-70.2)	50.8 (37.3-70.3)	53.7 (43.8-64.5)	56.1 (43.4-65.9)	54.0 (47.8-67.2)	54.2 (38.4-63.8)	59.7 (46.2-72.9)
MCFA	47.0 (23.7-58.9)	36.2 (23.5-45.3)	24.3 (10.7-43.4)	25.9 (8.67-45.7)	26.9 (17.1-38.3)	19.3 (10.7-31.4)	20.9 (13.0-26.3)	23.0 (14.5-41.3)	34.6 (19.8-54.3)
16:1ω7	2.87 (1.56-6.59)	3.38 (1.62-6.39)	2.18 (0.81-6.23)	1.35 (0.82-2.33)	2.65 (1.44-5.55)	3.03 (1.68-5.29)	2.19 (1.11-4.14)	1.47 (0.67-2.57)	1.95 (1.30-5.04)
18:1ω7	1.59 (1.07-3.72)	1.98 (1.09-3.33)	1.41 (0.81-2.76)	2.67 (1.00-6.30)	1.34 (0.92-2.93)	1.68 (1.05-2.54)	1.53 (1.09-2.55)	2.77 (1.12-4.64)	1.63 (0.58-4.91)
ω7	4.44 (2.79-10.1)	5.62 (2.81-9.20)	3.67 (1.65-8.31)	3.92 (1.82-8.63)	3.89 (2.37-8.37)	4.78 (2.89-7.88)	3.73 (2.22-6.74)	4.25 (1.83-7.18)	4.23 (1.97-8.01)
18:1ω9	12.8 (8.13-24.3)	16.1 (10.2-25.3)	25.8 (15.5-32.3)	20.2 (9.90-32.7)	21.2 (14.9-29.7)	27.7 (18.8-34.4)	27.8 (22.9-32.1)	24.8 (13.9-28.6)	19.4 (9.06-26.1)
20:1ω9	0.19 (0.00-0.32)	0.04 (0.00-0.26)	0.27 (0.03-0.53)	0.24 (0.09-0.55)	0.13 (0.00-0.38)	0.29 (0.00-0.40)	0.29 (0.00-0.39)	0.28 (0.13-0.40)	0.18 (0.08-0.31)
24:1ω9	0.06 (0.03-0.14)	0.07 (0.05-0.15)	0.08 (0.04-0.14)	0.07 (0.04-0.12)	0.09 (0.05-0.12)	0.05 (0.03-0.09)	0.08 (0.04-0.09)	0.08 (0.04-0.11)	0.00 (0.00-0.06)
ω9	13.2 (8.39-24.7)	16.4 (10.5-25.7)	26.2 (15.9-33.0)	20.7 (10.1-33.4)	21.6 (15.1-29.9)	28.2 (19.1-35.0)	28.4 (23.3-32.6)	25.2 (14.2-29.3)	19.8 (9.22-26.5)
MUFA	17.9 (11.9-34.2)	22.5 (14.8-35.1)	30.1 (18.2-37.8)	24.7 (14.1-40.8)	25.5 (18.6-38.2)	33.9 (22.6-43.1)	32.2 (27.2-38.5)	30.2 (16.2-33.8)	24.8 (11.3-33.5)
18:3ω3	0.28 (0.14-0.91)	0.53 (0.29-2.52)	0.77 (0.30-1.40)	1.27 (0.35-2.13)	0.53 (0.26-1.05)	0.63 (0.28-1.01)	0.78 (0.48-1.90)	0.85 (0.29-1.92)	0.90 (0.48-2.04)
20:5ω3	0.13 (0.06-0.29)	0.41 (0.05-0.98)	0.07 (0.02-0.68)	0.19 (0.03-0.41)	0.06 (0.02-0.13)	0.07 (0.03-0.12)	0.06 (0.04-0.13)	0.06 (0.02-0.12)	0.07 (0.00-1.37)
22:5ω3	0.23 (0.15-0.44)	0.68 (0.17-1.98)	0.17 (0.09-0.56)	0.43 (0.10-0.82)	0.19 (0.08-0.25)	0.22 (0.10-0.42)	0.18 (0.16-0.35)	0.17 (0.06-0.31)	0.15 (0.00-0.27)
22:6ω3	0.73 (0.35-1.38)	1.79 (0.53-3.37)	0.53 (0.20-1.58)	0.91 (0.16-1.88)	0.30 (0.13-0.76)	0.20 (0.11-0.51)	0.25 (0.14-0.55)	0.16 (0.07-0.36)	0.22 (0.13-0.51)
LCPω3	1.08 (0.56-1.99)	2.90 (0.75-6.28)	0.75 (0.32-2.82)	1.48 (0.34-3.12)	0.55 (0.28-1.14)	0.51 (0.26-0.80)	0.48 (0.36-1.03)	0.41 (0.19-0.61)	0.52 (0.13-1.83)
ω3	1.57 (0.82-2.21)	3.35 (1.22-6.83)	1.50 (0.88-3.83)	2.55 (0.89-4.59)	1.12 (0.54-1.62)	1.11 (0.53-1.68)	1.34 (0.91-2.81)	1.21 (0.48-2.53)	1.37 (0.61-2.49)
18:2ω6	4.23 (2.64-7.54)	5.20 (2.42-11.7)	9.91 (4.65-20.9)	18.1 (10.0-30.2)	14.5 (7.57-27.5)	8.79 (6.40-16.2)	11.1 (3.04-15.6)	15.5 (7.88-25.1)	14.3 (5.90-25.5)
20:4ω6	0.50 (0.36-0.73)	0.58 (0.43-1.06)	0.50 (0.37-0.94)	0.80 (0.53-1.07)	0.71 (0.44-0.94)	0.37 (0.27-0.57)	0.45 (0.41-0.58)	0.64 (0.32-0.94)	0.59 (0.39-0.83)
22:4ω6	0.14 (0.09-0.27)	0.14 (0.09-0.44)	0.12 (0.08-0.24)	0.20 (0.11-0.35)	0.20 (0.11-0.28)	0.08 (0.05-0.13)	0.10 (0.07-0.14)	0.16 (0.08-0.27)	0.13 (0.09-0.15)
22:5ω6	0.09 (0.05-0.13)	0.14 (0.05-0.36)	0.07 (0.04-0.50)	0.13 (0.03-0.21)	0.08 (0.04-0.12)	0.03 (0.01-0.06)	0.04 (0.00-0.06)	0.08 (0.03-0.12)	0.08 (0.00-0.09)
LCPω6	1.14 (0.87-1.50)	1.30 (0.94-2.53)	1.25 (1.02-2.40)	2.22 (1.47-3.14)	1.97 (1.41-2.55)	1.04 (0.71-1.66)	1.20 (0.85-1.67)	1.83 (0.95-2.74)	1.57 (0.92-1.73)
ω6	5.38 (3.82-8.83)	6.68 (3.91-13.4)	11.6 (6.09-22.5)	20.5 (12.2-32.6)	16.7 (9.08-29.8)	9.91 (7.28-18.0)	12.5 (4.19-16.9)	17.4 (8.88-27.7)	16.1 (6.88-27.3)
PUFA	6.86 (5.22-10.5)	10.7 (6.93-16.9)	13.0 (7.13-23.5)	24.3 (14.8-33.5)	17.9 (9.70-31.6)	10.8 (8.36-19.2)	13.8 (5.57-17.9)	18.7 (10.2-28.8)	18.1 (8.30-28.5)
AA/DHA	0.65 (0.49-1.12)	0.32 (0.24-0.90)	1.04 (0.36-2.44)	0.86 (0.51-4.49)	2.24 (0.76-6.40)	2.12 (0.71-3.44)	2.01 (0.74-3.48)	3.28 (2.27-8.61)	2.07 (1.41-4.42)
EPA/DHA	0.17 (0.09-0.22)	0.23 (0.10-0.48)	0.14 (0.08-0.43)	0.21 (0.11-0.44)	0.20 (0.08-0.43)	0.36 (0.15-0.62)	0.26 (0.11-0.39)	0.35 (0.11-0.86)	0.27 (0.00-7.32)
12:0/14:0	0.92 (0.63-1.34)	0.62 (0.32-0.84)	0.77 (0.45-1.08)	0.90 (0.51-1.39)	0.78 (0.40-1.18)	0.82 (0.58-1.04)	0.79 (0.51-1.00)	0.83 (0.61-1.07)	1.15 (0.84-1.61)

Data are median (range)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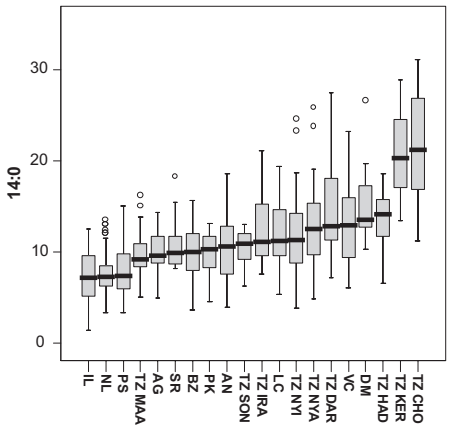
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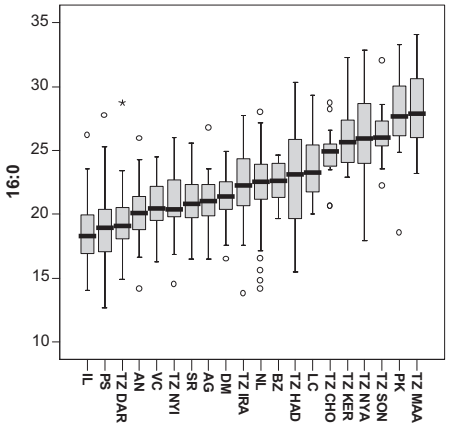
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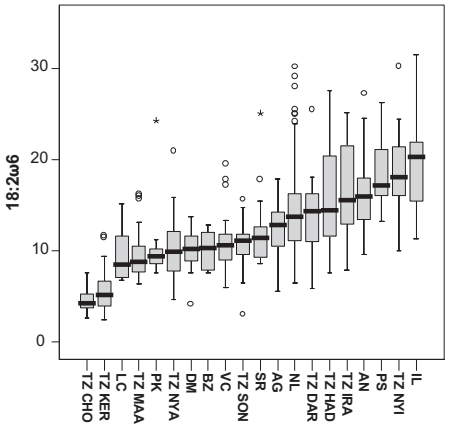
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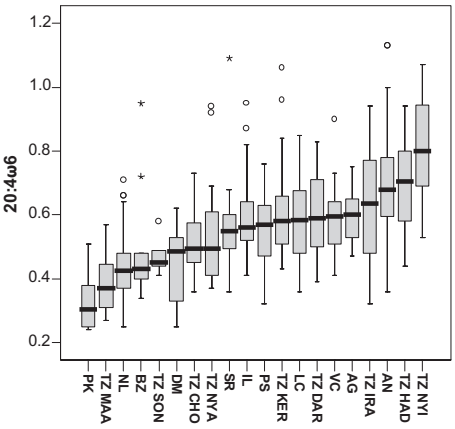
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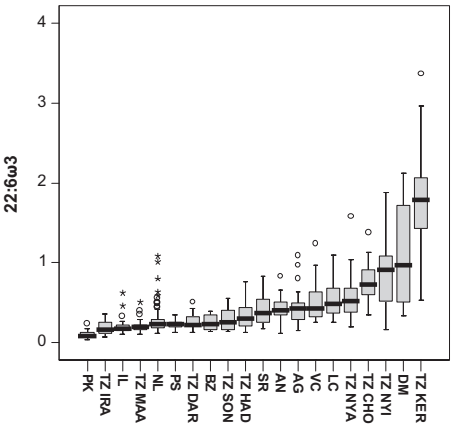
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Figure 1. Selected fatty acids in the milk of Tanzanian tribes and locations, as compared with countries in our world data set. Data (in g%) are indicated as box plots, showing medians (black bar), interquartile range (box length), minimum and maximum of the sample or smallest and largest values inside a 'reasonable' distance from the end of the box (crossbars at the far ends of whiskers), values between 1.5-3 box lengths from either end of the box (outliers, indicated by °) and values >3 box lengths from either end of the box (extremes, indicated by *).

Countries were denoted according to ISO 3166-1 and the corresponding ISO 3166-1-alpha-2 code elements for countries. Investigated tribes and locations in Tanzania are denoted as TZ, followed by the first three letters of the tribe or alternatively by the first three letters of the sampling location in case the local population has become mixed due to 19th century gathering of people from all over East-Africa, such as notably has occurred in coastal regions. AG, Antigua; BZ, Belize; DM, Dominica; IL, Jerusalem-Israel; LC, St. Lucia; NA, Curaçao-Netherlands Antilles; NL, The Netherlands; PK, Pakistan-Islamabad; PS, Jerusalem-Palestine; SR, Surinam; TZ-CHO, Chole; TZ-DAR, Dar-es-Salaam; TZ-HAD, Hadzabe; TZ-IRA, Iraqw; TZ-KER, Kerewe; TZ-MAA, Maasai; TZ-NYA, Nyakius; TZ-NYI, Nyarimba; TZ-SON, Sonjo; VC, St. Vincent.

of 12:0 were also observed in our world data set for the Caribbean islands of Dominica, St. Vincent and St. Lucia and for Surinam (Figure 1). Ninety percent of the samples from Chole exceeded the ≤15 g% recommendation for formula milk, whereas for the Kerewe this amounted to 10% (Table 3). An apparently similar picture emerged for 14:0, which is less abundant in coconut compared with 12:0. Also here Chole scored highest (Table 1, 21.19 g%), but this time followed by the Kerewe (20.26 g%), who do not consume coconuts but have high carbohydrate intakes. The 12:0/14:0 ratio in coconuts is about 2.61 g/g, while this ratio in the milk from Chole, Dar-es-Salaam and the Kerewe amounted to 0.92, 1.15 and 0.62, respectively. These ratios were statistically significant by Mann Whitney U-tests (Chole vs Kerewe, $p<0.0001$; Chole vs Dar-es-Salaam, $p=0.045$; Kerewe vs Dar-es-Salaam, $p<0.0001$), in which the Kerewe ratio proved most distinctive. Eighty-five and 87% of milk from Chole and the Kerewe, respectively, did not comply with the recommendation of ≤ 15 g% 14:0, while 26-100% of the samples from the various Tanzanian groups did not comply with the (12:0 plus 14:0) ≤ 20 g% criterion (Table 3). In contrast, none of the 222 milk samples from The Netherlands in our world data base exceeded the ≤ 5 g% recommendations for 12:0 or 14:0.

Highest 16:0 was encountered in the Maasai (Table 1, 27.90 g%), followed by Pakistan (Figure 1). For the Maasai this observation relates to their consumption of fermented milk, while the high 16:0 in Islamabad-Pakistan may be caused by the consumption of ghee[22]. Both milk and butterfat are abundant in 16:0.

Milk LA contents (Figure 1) were low in both Chole (Table 1, 4.23 g%) and the Kerewe (5.20 g%), notably when compared with the Nyiramba (18.11 g%) and the Iraqw (15.52 g%) who use abundant corn oil for frying and cooking. Also the Maasai and the Nyakius had relatively low milk LA, but most of the Tanzanian groups exhibited LA levels comparable with the affluent countries in our world data set.

Highest levels in the latter were reached by Jerusalem-Israel (Figure 1). Sixty-five and 47 percent of Chole and the Kerewe samples, respectively, did not comply with the LA ≥ 5 g% recommendation, while 100 and 93% did not comply with LA ≥ 11 g% (Table 3). The Nyiramba, Hadzabe, Sonjo and

Table 3. Percentages of human milk samples that do *not* fulfill the recommendation as issued by the indicated organization

committee	recommendation	CHO	KER	NYA	NYI	HAD	MAA	SON	IRA	ALL
CD91, EU	12:0<=15 g%	90	10	3	3	4	0	0	11	13
CD91, EU	14:0<=15 g%	85	87	27	17	32	7	0	28	37
EU, ESP05	12:0+14:0<=20 g%	100	100	63	63	86	26	33	50	68
ESP05	18:2ω6>=5 g%	65	47	3	0	0	0	11	0	15
LSRO, EU	18:2ω6>=8 g%	100	87	27	0	4	33	22	6	34
CD91	18:2ω6>=9 g%	100	90	37	0	4	56	22	11	40
GR, WS	18:2ω6>=10 g%	100	93	50	0	14	67	33	22	47
FAO, ESP	18:2ω6>=11 g%	100	93	63	3	21	81	44	22	53
ESP, CD91	18:2ω6<=20 g%	0	0	3	46	32	0	0	33	16
ESP, EU	18:2ω6<=27 g%	0	0	0	3	4	0	0	0	1
LSRO	18:2ω6<=35 g%	0	0	0	0	0	0	0	0	0
FAO, ESP05, EU	18:3ω3>=1.0 g%	100	93	73	37	96	96	78	78	80
GR	18:3ω3>=1.4 g%	100	97	97	66	100	100	89	89	91
SI, WS	18:3ω3>=1.5 g%	100	97	100	69	100	100	89	89	92
LSRO	18:3ω3>=1.75 g%	100	97	100	83	100	100	89	89	95
LSRO	18:3ω3<=4.0 g%	0	0	0	0	0	0	0	0	0
CHF, EU	20:4ω6>=0.35 g%	0	0	0	0	0	41	0	6	7
WS	20:4ω6>=0.5 g%	50	20	53	0	7	85	89	33	37
GR	20:4ω6>=0.6 g%	85	50	73	14	36	100	100	44	58
FAO	20:4ω6>=0.8 g%	100	87	93	49	75	100	100	78	82
SI	20:4ω6<=1.0 g%	0	3	0	17	0	0	0	0	4
WS	20:5ω3<=0.10 g%	70	97	20	71	14	11	22	11	43
CHF, EU	22:6ω3>=0.2 g%	0	0	3	6	25	56	44	67	22
WS	22:6ω3>=0.35 g%	0	0	23	20	57	93	56	94	40
FAO, GR	22:6ω3>=0.4 g%	5	0	27	20	68	93	78	100	44
CD96, SI, EU	20:5ω3<=22:6ω3	0	0	0	0	0	0	0	0	0
EU	LCPω6<=22:6ω3	5	93	3	0	0	0	0	0	15
SI, ESP, CD96, EU	LCPω6<=2 g%	0	3	3	83	46	0	0	28	25
SI, ESP, CD96, EU	LCPω3<=1 g%	60	97	23	71	4	0	11	0	38

Cho, Chole (n=20); Ker, Kerewe (n=30); Nya, Nyakius (n=30); Nyi, Nyiramba (n=35); Had, Hadzabe (n=26); Maa, Maasai (n=27); Son, Sonjo (n=9); Ira, Iraqw (n=18); All (n=197) CD91, Commission Direcetive 1991; CD96, Commission Directive 1996; CHF, Child Health Foundation, 2001; ESP, ESPHGAN 1991 and 2005; EU, European Commission, Scientific Committee on Food 2003; FAO, Food and Agriculture Organization of the United Nations, 1994; GR, Health Council of the Netherlands (Gezondheidsraad), 2001; LSRO, Life Science Research Office, 1998; SI, Statutory Instruments, 2001; WS, Workshop Statement, 2000. LCP, long-chain polyunsaturated fatty acids

Iraqw were the only groups in which over 50% fulfilled the LA ≥ 11 g% recommendation, whereas for The Netherlands this figure amounts to 76%[28].

Although their LA was low, AA in milk from the fish-eating Chole mothers (Table 1, 0.50 g%) was comparable with AA in both the milks of the fish-eating Nyakius (0.50 g%) and Kerewe (0.58 g%), but much lower than in the fish-eating Nyiramba (0.80 g%). From the groups living in the inland, the Hadzabe show highest AA (0.71 g%), while the Maasai (0.37 g%) had levels comparable with Pakistan and The Netherlands (Figure 1). Except for the Maasai, all milk samples in Tanzania fulfilled

the ≥ 0.35 g% AA recommendation (Table 3), while in The Netherlands 14% do not comply (28). Forty-one percent of the Maasai did not reach the ≥ 0.35 g% AA recommendation, while 51% of the samples from the fish-eating Nyiramba reached the AA ≥ 0.8 g% criterion (Table 3). None of the samples in The Netherlands reached the AA ≥ 0.8 g% criterion.[28]

Three out of the 4 investigated fish-eating groups showed high EPA percentages that did not fulfill the ≤ 0.1 g% recommendation. The percentages that did not comply amounted to 97, 71 and 70% for the Kerewe, Nyiramba and Chole groups, respectively (Table 3). In the Netherlands only 9% did not comply to this criterion.[28]

The 4 fish-eating groups had highest milk DHA levels, ranging from 0.53 g% for the Nyakius up to 1.79 g% for the Kerewe, and with Chole (0.73 g%) and the Nyiramba (0.91 g%) holding intermediate positions (Table 2). These levels were well above those of the groups living in the inland, with the vegetarian Iraqw (0.16 g%) and the pastoral Maasai (0.20 g%) showing lowest levels. Many of the groups living in the inlands showed DHA levels comparable with affluent countries such as The Netherlands (Figure 1). Five and 0% of the samples from Chole and the Kerewe, respectively, did not comply with the DHA ≥ 0.40 g% recommendation, whereas for The Netherlands and Jerusalem-Palestine these figures amount to 92 and 97%, respectively.[28]

Figure 2 compares the AA/DHA ratios in the milk of the 4 fish-eating groups with the corresponding AA/DHA ratios in the fishes that are available to them. Neither of the locations

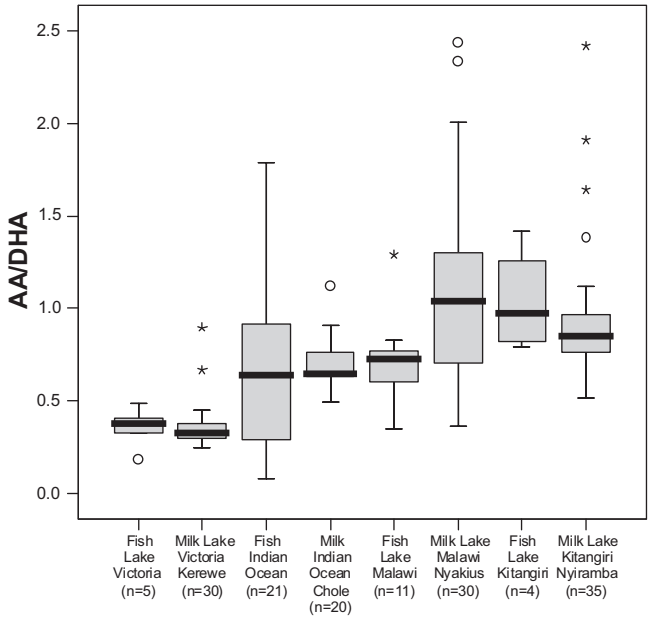

Figure 2. Comparison between AA/DHA ratios in milk and fish. Data (in g/g) are presented as box plots (see legend of Figure 1). They derive from the Kerewe (Lake Victoria), mothers living in Chole (Indian Ocean), the Nyakius (living at Lake Malawi) and the Nyiramba (living at Lake Kitangiri). AA, arachidonic acid (20:4ω6); DHA, docosahexaenoic acid (22:6ω3).

showed differences between the fish and milk AA/DHA ratios. However, except for the comparison between Lake Malawi and Lake Kitangiri, there were significant differences between the milk AA/DHA ratios for the various locations (Lake Victoria vs Indian Ocean $p<0.0001$, Lake Victoria vs Lake Malawi $p<0.0001$, Lake Victoria vs Lake Kitangiri $p<0.0001$, Indian Ocean vs Lake Malawi $p=0.003$, Indian Ocean vs Lake Kitangiri $p=0.003$.

The AA/DHA ratio in fish does not seem dependent on their derivation from fresh or saltwater, since there were significant differences between the AA/DHA ratios of the fish from Lake Kitangiri and Lake Victoria ($p=0.042$) and Lake Kitangiri and Lake Malawi ($p=0.024$). The largest variation of the AA/DHA ratio was observed in fish from the Indian Ocean, which may relate to the wide variety of marine locations from which the investigated fishes originated.

DISCUSSION

There is no 'uniformity of human milk' and this applies notably for the human milk fatty acid composition.[8,79] Within the limitations of the genetically-determined preferences within the mammary gland triacylglycerol assembly machinery, the human milk fatty acid composition seems predominantly dependent on the fatty acid composition that is submitted to this machinery. Strong preferences for incorporation of certain fatty acids at certain sn-positions of the glycerol backbone may provide some structural uniformity, such as the high preference to incorporate 16:0 on sn-2,[80] but the dietary composition and to a lesser extent maternal extra-mammary physiology are by far the most important modifiable factors of the total milk fatty acid composition. Consequently, the large variance in the world-wide human milk fatty acid compositions F, but also within a single country such as Tanzania (Table 2), is testimony of the wide varieties of foods that are tolerated by human beings, with dietary fatty acid composition and macronutrient composition (notably carbohydrate content) as major determinants. This remarkable flexibility is however not to be taken as proof for unimportance, since criticizing the current Western diet for its ability to cause diseases typical for affluent societies might be a synonym for criticizing the current Western milk fatty acid composition. Concerns regarding adequate intakes of LCP by neonates to support optimal brain development are an example[81-83], while also the consistently found epidemiological relation between early environmental factors and adult disease[84-86] become rapidly supported by the elucidation of the underlying (epigenetic) mechanisms.[87,88]

In view of the overwhelming influence of the maternal diet, the question arises what human milk fatty acid composition supports infant development at best. Many randomized controlled trials with fatty acid-enriched formulae have been, and are presently, conducted, using many different endpoints, such as those of neurological development in case of LCP.[81-83] The limitations of these studies are that they are performed with infants that have been pre-exposed to an intrauterine environment that is characterized by Western dietary habits, that the tested LCP contents are based on their contents in milk from Western mothers, that use of formula precludes investigation of interaction of the supplement with human milk constituents, while it is virtually impossible to study

all potential interactions by performing trials with formulae. Consequently, randomized controlled trials with formula-fed Western infants, using LCP contents as encountered in human milk from Western mothers, will at most confirm 'optimality' of Western human milk and will subsequently lead to recommendations based on the underlying 'evidence of optimality'. In other words, human milk is not a popular subject to become questioned for its optimality. To avoid this vicious circle and to limit laborious dose-response studies and studies addressing interactions, we seem to be in need of some 'clues' for the optimal milk fatty acid composition, which by definition has been established by millions of years of evolution occurring in the context of a more constrained diet as presently consumed worldwide.

In search of such clues we previously reported the human milk fatty acid composition of Tanzanian women consuming freshwater fish as the only source of animal protein and fat;[59] (see Table 2, Nyiramba). Given the notion that *Homo sapiens* evolved in such eco-systems, we suggested that their high milk AA and DHA contents might closely reflect our ancient diet. On the other hand, the Nyiramba diet was also composed of high carbohydrates and refined vegetable oils, which were unavailable to our ancient ancestors. High carbohydrate intakes, causing high milk MCSAFA contents, and abundant use of LA-rich vegetable oils were however unlikely to influence milk AA and DHA, since there is no appreciable competition between LCP on the one hand and MCSAFA and LA on the other, for their incorporation into milk triacylglycerols[59]. The Nyiramba milk may consequently have provided valuable information on the LCP status of ancient communities, but failed to provide clues for the intakes of carbohydrates and LA. In the present study we extended our search for the evolutionary established human milk fatty acid composition by studying the milk from Chole. The Chole inhabitants combine animal protein and fat from the local land-sea interface with vegetables, fruits and coconuts. Their milk is characterized by a high DHA content (0.73 g%), which is comparable with milk from the freshwater fish eating Nyakius (0.53 g%) and Nyiramba (0.91 g%), but was well below that of the extremely high DHA levels encountered in the freshwater fish eating Kerewe (1.79 g%). Milk DHA of Kerewe mothers was even higher than the DHA content that was previously reported for Inuit (38) milk (i.e. 1.4 g%). The Kerewe milk DHA content was, however, not as high as that of fish eating Chinese mothers (i.e. 2.8 g%),[89] while the Inuit milk EPA content was higher than that of Kerewe mothers (Inuits: 1.1 g%; Kerewe 0.41 g%)[38]. The high milk DHA in Chole could be traced to the consumption of marine fish and shows that abundant consumption of both marine and freshwater fish gives rise to milk DHA contents that are well above those encountered in people living in the Tanzania inlands, most Western countries and the recommendations derived from the latter (Figure 1, Table 3). Regarding its AA content, Chole milk seems to hold an intermediate position among both Tanzanian fish-eating populations and those living in the inlands. Chole median milk AA levels (0.50 g%, Table 2) are clearly higher than those of the Maasai (0.37 g%) but comparable with those encountered in a country like The Netherlands (0.43 g%). All Chole samples showed AA levels above the 0.35 g% recommendation, whereas for the Maasai and The Netherlands 41% and 14%[28] of samples did not comply with this criterion, respectively.

The milk fatty acid composition of Chole also reminds us of the tremendous increase of LA consumption in past decades that seems subsequently to have become adopted as the basis for LA intake from infant formulae. Newborns in Chole do not show the skin problems associated with LA deficiency, which relates to the unique function of LA in skin ceramides.[90] Moreover, milk LA contents in the USA prior to augmentation of LA intake were typically in the 5-7 g% range.[91] Interestingly, only 35% of the Chole samples complied with LA >5 g%, whereas none of them complied with LA >8 g% and consequently the even higher recommendations (Table 3). Lower LA in milk from Chole seems predominantly caused by the almost nonexistent use of LA-rich vegetable oils for cooking, but may in part also be caused by the high consumption of coconuts. Ingestion of a single dose of 12:0-rich coconut oil has previously been shown to rapidly augment the milk 12:0 content with peak concentrations being reached after 12-14 h (70). Both 12:0 and 14:0 compete with LA for incorporation into milk triacylglycerols.[59] High milk 12:0 and 14:0 from coconut oil seems to some extent distinguishable from similar increases as derived from a carbohydrate-rich diet. Indications for this may be taken from the higher 12:0/14:0 ratio in Chole, as e.g. compared with the corresponding ratio in the milk of the Kerewe (0.92 vs 0.62 g/g). The Kerewe population does not have access to coconuts or coconut oil, and their high 12:0 and 14:0, but lower 12:0/14:0, derives almost exclusively from carbohydrate consumption. High 12:0/14:0 ratio as deriving from coconut consumption became confirmed from the historical milk data from Dar-es-Salaam (12:0/14:0 1.15 g/g), since in Dar-es-Salaam coconuts are both consumed and its oil used for cooking.

A high milk MCSAFA might have been part of the ancient diet of newborns living in tropical coastal areas, where coconuts were abundant. It is however unlikely that abundant dietary carbohydrates have in the past served as substrates for mammary gland MCSAFA production to the extent as observed in the Kerewe and Nyiramba, since carbohydrate-rich diets were not introduced until the start of the agricultural revolution, some 10,000 years ago. Milk MCSAFA may confer many favorable properties to the newborn. Their contents increase with advancing lactation,[80,92] they serve as easily absorbable energy sources, and they exhibit broad-spectrum antiviral and anti-microbial properties.[93-95] These anti-microbial effects seem notably on account of 8:0 and 12:0, and to a much lesser extent on 6:0, 10:0 and 14:0.[95] Interestingly, both the milks from coconut-eating Chole and Dar-es-Salaam exhibited the highest 8:0 and 12:0 contents (Table 2). The atherosclerotic effect of coconut oil in adults, is uncertain, but at least its effect on cholesterol deserves some nuance.[96] Both 12:0 and 14:0 raise LDL-cholesterol, but they also decrease the total cholesterol/HDL-cholesterol ratio. Actually, 12:0 (lauric acid) has a more favorable effect on the total cholesterol/HDL-cholesterol ratio than any other fatty acid, either saturated or unsaturated, while the corresponding effect of the 2.6 times less abundant 14:0 (myristic acid) in coconuts is less pronounced. In contrast, on an isocaloric basis, carbohydrates induce the highest increase in total cholesterol/HDL[96] which suggests an atherosclerosis promoting effect of the carbohydrate-rich diet in the Kerewe adult population, but also in their offspring because of the carbohydrate-induced lower 12:0/14:0 ratio in their milk, compared with Chole. Finally, coconut water is isotonic[97] and provides an excellent

source of sterile fluid to compensate for losses in a hot tropical climate with little freshwater in the immediate surroundings.

In summary, the human milk fatty acid composition in Chole has provided valuable information on the possible dietary composition of babies born to our ancient East-African ancestors living in coastal areas. Compared with current Western counterparts, their diet might have been abundant in AA, DHA and MCSAFA (notably 12:0 and 14:0) and poor in LA. Whether this composition supports optimal homeostasis and exerts antimicrobial effects is conceivable, but should be tested in randomized controlled trials using clearly defined end points. The apparent lack of adverse effects in the children in Chole, but also in other children living in Tanzanian land-water interfaces, seems to argue against the validity of current recommendations that are merely based on the human milk composition of Western mothers.

Acknowledgments

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CHAPTER 3.3

The basis of recommendations for docosahexaenoic (DHA) and arachidonic (AA) acids in infant formulae: absolute or relative standards?

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Dear Sir:

Brenna et al.[98] provided us with a meta analysis on the worldwide human milk DHA and AA contents. Their study was composed of 65 reports of 2474 women with mostly Western dietary habits. Analogous to at least two previous studies[8,99] they concluded that milk DHA is more variable than milk AA and that the DHA/AA ratio varies widely. The calculated mean±SD values for DHA (0.32±0.22 g%) and AA (0.47±0.13 g%) were suggested to serve as a guide for infant feeding.

Although their conclusion on variation is consistent with one of our previous reports[8] we currently feel that the low AA variation might in reality derive from a sampling bias. Much higher milk AA (median 0.70 mol%) together with high DHA (0.75 mol%) was found by us in lactating women in Doromoni (Tanzania).[59] Their milk showed clear correlation between DHA and AA. Both of these long chain polyunsaturated fatty acids (LCP) could be traced to life-long consumption of DHA- and AA-rich fish from the nearby freshwater lake Kitangiri. It seems that milk AA is notably dependent on long term AA intake, since supplementation of lactating women in Jerusalem with 300 mg AA for one week did not increase milk AA.[22] The sizeable short and long term dietary influences on the human milk fatty acid composition raise the question whether the resulting variance should be taken as testimony of the wide variety of foods that are tolerated by human beings and their offspring, or that it indicates a lack of evolutionary pressure because of a rather constant dietary composition in the past. We feel that the worldwide human milk fatty acid composition should not be taken as guide for infant formula. As much as Western serum cholesterol levels and vitamin D status should not serve as targets for recommendations, it seems inappropriate to take the calculated mean milk DHA and AA of mostly Western women as a basis for infant formulae. At least three arguments are in favor of higher intakes of both DHA and AA by our ancient ancestors, consuming diets that were much closer to the environment on which our genome has become adapted during the past 2-3 million years of evolution. *First,* the sites at which their fossil remains have been discovered are in support of the notion that evolution to *Homo sapiens* took place on an LCPω3-rich diet from East-African ecosystems that were located in places where the land meets with water.[16,100] Food from these ecosystems is rich in iodine, vitamins A and D, and ω3-fatty acids from both vegetable origin and fish. Contrary to popular believe, our ancient ancestors did not need fishing gear to benefit from the abundant LCPω3, and probably LCPω6, in such ecosystems, where it is relatively easy to hunt and gather anything ranging from spawning (cat)fish, shellfish, mollusks, jellyfish, lobsters, eggs, birds and reptiles, which all ultimately receive their LCPω3 from plankton via the local food-chain. The resulting high iodine, vitamins A and D, and LCP diet seems somewhat abandoned since the Out-of-Africa Diaspora, since deficiencies of these nutrients are among the most widely encountered in the current world population.[16,52] *Second,* epidemiological data demonstrated a negative association of fish oil with coronary artery disease (CAD), and of fish consumption with (postpartum) depression. Landmark trials with alpha-linolenic acid (ALA),[57] fish oil[101] and eicosapentaenoic acid (EPA)[102] in CAD, and with EPA in depression and schizophrenia[58] supported the causality of these relations. As acknowledged by Brenna et al.[98] the intake of marine food in inland and developed countries is

usually low. Many authorative organizations issued recommendations to the general public ranging from 'choose fish as a food item more often' up to 'the consumption of three servings of fish per week'. One may wonder what the milk DHA and resulting DHA status would be of children born to parents reaching the necessary DHA status to decrease their risks of CAD and psychiatric disease. If their parents would benefit from higher than present Western DHA status, which cannot be achieved by the mere consumption of ALA, it seems reasonable to assume that this DHA status is appropriate across the *Homo sapiens* lifecycle and that also our genes may have evolved on this high DHA status. Meanwhile, it has also become clear that LCP are not only important structural components of membranes but, together with their eicosanoid metabolites, firmly implicated in gene expression, e.g. as modulators of nuclear transcription factors such as PPARs, SREBPs and TNF-alpha. *Finally*, many randomized controlled trials using formulae with and without LCP, and measuring outcome parameters like retinal function, visual acuity, behavior, and cognitive and motor developments, have shown beneficial effects of LCP, notably LCPω3, in both preterm and term infants. Preterms benefit most, but many of the effects are transient. Effects are especially on account of DHA, but addition of AA might be important to preserve ω3/ω6 balance. The present consensus from human and animal studies is that LCP supplements have no effect on growth, that neonatal brain DHA is positively related to cognitive and behavioral performance, that the differences are difficult to detect with currently available tools, but that the encountered differences may nevertheless be relevant.[103] All of these studies have been performed with DHA and AA dosages in the current 'Western' human milk range, which might explain the modest effects.

In conclusion, we agree that DHA seems the most variable fatty acid in the human milk worldwide. AA variance may, however, be underestimated due to a sampling bias. Higher AA levels occur in women consuming diets close to our ancient diet, which is part of the environment on which our genes evolved. Current Western human milk DHA contents do not comply with recommendations of authorative organizations issuing advises to increase our fish intake and should consequently not serve as guide for infant feeding. It would be of interest to see whether infant formulae with LCP dosages consistent with non-Western, traditionally, eating populations would produce more pronounced effects using the many endpoints that have been studied with limited success until now.

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CHAPTER 3.4

Fatty acid compositions of preterm and term colostrum, transitional and mature milk in a population with high fish intakes

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Prostaglandins, Leukotrienes and Essential Fatty Acids, in press

ABSTRACT

Background. Lower long-chain polyunsaturated (LCP) fatty acid (FA), notably docosahexaenoic acid (DHA), in term compared to preterm milks is often attributed to more depleted maternal LCP stores after full term gestation. Depletion of maternal DHA stores is preventable by high maternal DHA-intakes.

Objective: Investigating FA differences between preterm and term milks.

Methods. We compared the FA-compositions of preterm (28-36 weeks) and term (37-42) colostrum (2-5 days), transitional (6-15) and mature (16-56) milks in African women with stable dietary habits and lifelong high freshwater fish intakes.

Results. From colostrum to mature milk, the median DHA and arachidonic acid (AA) contents decreased from 1.11-0.75, and 0.93-0.69 g% in preterm; and from 0.81-0.53 and 1.08-0.55 g% in term milk, respectively. Medium-chain FA (MCSAFA) increased from 16.9-33.7, and 7.92-29.0 g%, while mono-unsaturated FA (MUFA) decreased from 32.5-22.6, and 40.0-26.5 g%, in preterm and term milk, respectively. Consistent with the literature, preterm colostrum contained higher DHA and MCSAFA, and lower MUFA compared to term colostrum. These differences vanished rapidly with advancing lactation. MUFA and MCSAFA were inversely related.

Conclusions. African women show much higher milk MCSAFA and lower MUFA contents, compared to European counterparts. DHA in preterm milks proved the highest reported so far. DHA differences between preterm and term colostrum are unlikely to originate from maternal depletion between preterm and term delivery. We hypothesize that changes and differences in insulin sensitivity may contribute to the changing FA composition with advancing lactation and to the FA differences between preterm and term colostrum.

INTRODUCTION

Human milk is the optimal nutrition for the newborn. The milk fatty acid (FA) composition results from some combination of FA uptake from triglycerides (TG) in circulating VLDL and chylomicrons by lipoprotein lipase (LPL),[104] FA uptake from circulating albumin-bound free-FA[105] and endogenous *de novo* lipogenesis from glucose in the breast. It was estimated that 10-12% of milk lipids are derived from *de novo* lipogenesis in the mammary gland, that 29% originate directly from the diet and that 60% are derived from maternal stores (104). The essential FA, alpha-linolenic and linoleic acid may originate for 30% directly from the diet and for 70% from maternal depots.[106] The human milk lipid content varies during the course of a feeding, during the day and longitudinally from delivery till weaning, but the FA composition seems only to be affected by time postpartum, gestational age, parity and disease.[107] The composition is also highly sensitive to changes in the maternal diet.[25,70,108] High intakes of dietary carbohydrates increase milk medium-chain saturated FA (MCSAFA) that are uniquely *de novo* synthesized in the mammary.[25,26] Consumption of tropical fish rich in both arachidonic (AA) and docosahexaenoic (DHA) acid increases both of these long-chain polyunsaturated FA (LCP) in milk lipids.[59,109,110] The worldwide human milk FA composition varies the most in DHA,[8,99,111] but is remarkably uniform in AA[98]

The milk LCP content decreases consistently with advancing lactation[92,112-119] and is consistently higher in preterm compared to term colostrum.[115,120-124] Comparably, human milk mono-unsaturated FA (MUFA) decrease quite consistently with advancing lactation,[92,112-114,118,119,123] while the MCSAFA content increases.[92,112-114,116-119,123] Early preterm milk contains lower MUFA[120,121,123,125] and higher MCSAFA[112,120,121,123,126] compared to early term milk, but few of these differences are still noticeable in more mature milk.[117] The decrease of milk LCP with advancing lactation and the lower content of LCP in term compared to preterm human colostrum milk has been ascribed to the depletion of maternal stores,[127] while decreasing MUFA has been attributed to increasing MCSAFA, secondary to the maturation of the mammary gland (105). The consistently observed differences for MUFA and MCSAFA between preterm and term colostrum remain largely unexplained, although Freed et al.[128] suggested that the higher MCSAFA in preterm compared to term colostrum might be attributed to higher *de novo* FA synthesis in the preterm mammary gland. Although some differences between preterm and term milk remain mysterious, the advantage of feeding preterm (donor) milk to premature infants has been advocated,[117,122,126] which is in line with the recommendation to supply the term newborn with human milk rather than infant formulae.[129]

We recently showed (130) that postpartum decreases in the maternal and infant erythrocyte (RBC)-DHA contents are dose-dependently abolished by high maternal intakes of tropical freshwater fish containing high amounts of AA and DHA.[111] More importantly, our data showed that in this population the maternal RBC-DHA content, a reliable index for DHA status, did not decrease during pregnancy, and that at delivery mothers with high DHA-status had even higher RBC-DHA contents compared to their infants, a process that we named bioattenuation[130,131] - as opposed to the biomagnification that takes place at lower DHA status.[132] We concluded that within this

unique population, living in Sengerema (Lake Victoria, Tanzania), there is no depletion of maternal DHA stores at least during pregnancy,[131] but that this occurs to some extent during subsequent lactation.[130]

We were interested to see whether the lifelong stable dietary habits, including relatively high intakes of carbohydrates and of DHA and AA by the women in Sengerema cause similar preterm-term differences in milk DHA, AA, MUFA and MCSAFA, as previously reported for other countries in which the populations usually eat much less fish. We were notably curious to see whether the absence of DHA depletion during pregnancy would equalize the DHA contents of preterm and term colostrum and thereby test the notion that the lower DHA in term colostrum is due to maternal DHA depletion in the period between preterm and term delivery.

SUBJECTS AND METHODS

Study design and study groups

The study was conducted in the Sengerema hospital, located at the southern shore of Lake Victoria in Tanzania. Although the area is densely populated, small villages are widely distributed throughout the landscape. Patients visit any nearby hospital or dispensary whenever their household work allows them or when their business brings them into the proximity. Since an initially started longitudinal study proved logistically almost impossible in this setting, we conducted a mostly cross sectional study in which we investigated the FA differences between colostrum (defined 1-5 days), transitional (6-14 days) and mature breast milk (>14 days) from women who delivered preterm (28-36 weeks of gestation) and at term (37-42 weeks). The local tribes are mostly Bantu and most families have been involved in fishing for centuries. Ugali (corn porridge), muhogo (cassava root) and plantain (baked banana) are staple foods, while both fruits and vegetables are eaten on a daily basis. Consumption of fish is frequent and one type of local fish (local name 'Dagaa', Scientific *Rastrineobola argentea*) is even regarded as a kind of vegetable. Since the hospital does not provide food to its patients, women bring their own food and cook it at the spot, after which it is shared with all neighboring persons. It is not a custom, nor is there any interest or actual possibility, to deviate from their usual diet during pregnancy or lactation. The average dietary intakes of mothers during lactation were thus likely to be representative for their lifetime dietary habits. Differences between dietary intakes of preterm and term delivering women are very unlikely.

All subjects were apparently healthy and well nourished, and had delivered their infants 2-56 days prior to the day of inclusion. Deliveries that might be related to disturbed maternal glucose homeostasis, e.g. preeclampsia and maternal (gestational) diabetes, were excluded. Preterm deliveries were mostly attributed to maternal infections, notably malaria. All mothers were exclusively breastfeeding and none of the infants received additional parenteral nutrition. All infants were permitted to suckle on demand and they consequently stimulated the mammary gland throughout the day. There were in this respect no differences between preterm and term delivering women, although milk for preterm infants was sometimes manually extracted and orally

administered, since the infants were unable to suckle for longer periods.

Data on age, parity, fish consumption and duration of lactation were obtained from the medical records or by interviews in Kiswahili, as conducted by local doctors or by one of us. Gestational ages were determined from the last known menstrual period (LMP). When the LMP was unknown we employed the fundal height (FH). When either the LMP or the fundal height indicated prematurity or term age, infants were routinely checked for the signs of prematurity. If no signs of prematurity were found in an infants previously classified as preterm (e.g. small for gestational age infants), or if these signs were observed in an infant previously classified as term (e.g. in polyhydramnios), their mothers were excluded from further analysis. All women provided informed consent. The study was approved by the National Institute for Medical Research in Dar-es-Salaam (NIMR/HQ/R.8a/Vol. IX/800, dated April 8, 2009) and was in agreement with the Helsinki declaration of 1975 as revised in 2000.

Samples and analyses

Although the milk lipid content does not affect the FA composition[107], milk collection was conducted uniformly for all groups. All samples were collected in the late morning, prior to consumption of the main meal around noon, when the MCSAFA content is relatively uninfluenced by the dietary carbohydrate intake.[26,108] Participating women were asked to briefly suckle their infant. After one minute, a milk sample of 2-5 ml was taken by manual expression from the same breast. An aliquot of 200 µL was transferred to a Sovirel tube containing 2 mL of methanol-6 mol/L HCl (5:1 by vol), 1 mg butylated hydroxytoluene (antioxidant) and 100 µg 5:0 up to 15:0 and 50 µg 17:0. In this ready-to-transmethylate mixture FA are stable at room temperature and in the dark for months.[29] All samples were transported at room temperature to the University Medical Center Groningen (the Netherlands) for FA analysis. Following transmethylation and extraction, analyses of FA methyl esters (FAME) were performed by capillary gas chromatography/flame ionization detection using a series of odd-chain numbered FA (5:0 up to 17:0) as internal quantification standards. MCSAFA (6:0 up to 14:0) were quantified with use of 5:0-15:0 as internal quantification standards.[30,78] Long-chain saturated FA (LCSAFA, ≥16:0) were quantified on the basis of the added 17:0. FA compositions and their ratios were expressed in g% and g/g. Data are presented as medians (minimum-maximum).

Statistics

Statistical analyses were performed with SPSS version 16.0.1 (SPSS Inc, Chicago, IL). Between-group differences for independent samples (i.e. preterm vs. term milks and term colostrum vs. term mature milk) were analyzed with the Mann Whitney U-test. For the comparison of preterm colostrum to preterm mature milk we had to remove 2 milk samples (1 colostrum sample and 1 mature milk sample) from the analysis, since these originated from 2 subjects that had been studied longitudinally (i.e. there were 2 women from whom we had collected a colostrum and a mature milk sample). After removal of these samples, the remaining independent samples were analyzed

milk sample). After removal of these samples, the remaining independent samples were analyzed with the Mann Whitney U-test. Corrections were made for type-1 errors according to Bonferroni. Correlations were investigated with linear regression analyses. Differences and correlations were considered significant at $p<0.05$. Data points in the figures represent the medians.

RESULTS

*S*tudy subjects, samples and characteristics

We included a total of 133 milk samples provided by the participants in this study. Term milk samples were from 83 different women at different stages of lactation and were collected within two weeks. We collected 30 colostrum, 19 transitional and 34 mature breast milk samples from women who delivered at term. Preterm milk samples were collected from 38 women. Taken together, we collected 14 preterm colostrum, 23 transitional and 13 mature preterm milk samples. These numbers do not sum up to 38, since some of the women who had delivered prematurely remained in the hospital. From 2 of these subjects we were able to collect longitudinal data for colostrum, transitional and mature milk, while 8 women provided either a colostrum and a transitional sample or a transitional and a mature milk sample. For statistical correctness (comparison of independent samples only), one of the samples of these subjects had to be excluded to compare preterm colostrum with mature milk. Although this methodology reduced the *n* in both groups (n-1); medians remained statistically indistinguishable; therefore these data are not presented separately.

The anthropometrics and other characteristics of the investigated mothers and their infants are shown in **Table 1**. At delivery, prematurely delivering women had a lower gestational age and their infants had lower birth weights. Women delivering at term had a higher parity, but this reached only significance for mothers breastfeeding >2 weeks. There were no other between-group differences for mothers who delivered prematurely and at term, except for a lower weight and BMI after 2 weeks of exclusive breastfeeding in women who had delivered prematurely.

Milk fatty acids

The FA compositions of preterm and term colostrum, transitional and mature breast milks are depicted in **Table 2** together with the directions of all significant changes.

Differences between preterm and term milk

SAFA and MUFA. **Figure 1** shows the apparent courses of milk MCSAFA and MUFA for both preterm and term delivering women. It must be noted that the depicted data are for colostrum, transitional and mature milk of predominately different women. Preterm colostrum had higher SAFA and MCSAFA (≤14C-atoms), specified by higher 8:0, 10:0, 12:0 and 14:0; but showed no difference with respect to LCSAFA (>14 C-atoms). Preterm colostrum exhibited lower 16:1ω7, 18:1ω7, ω7, 18:1ω9, 20:1ω9, ω9 and MUFA as compared to term colostrum. All differences between preterm and term milk SAFA, ω7 and ω9-FA, as observed in colostrum, disappeared with advancing lactation, with the

Table 1. Maternal and infant anthropometrics and characteristics[a,b]

	Preterm milk			Term milk		
	Colostrum *n = 14* mean ±SD	*Transitional* *n = 23* mean ±SD	*Mature* *n = 13* mean ±SD	*Colostrum* *n = 30* mean ±SD	*Transitional* *n = 19* mean ±SD	*Mature* *n = 34* mean ±SD
Maternal						
Age (years)	21.5 ±5.4	22.5 ±5.6	23.8 ±6.0	23.5 ±5.8	22.0 ±4.4	25.7 ±5.4
Length (cm)	154.3 ±6.0	153.1 ±5.4	152.0 ±5.3	154.4 ±5.5	156.4 ±4.6	155.5 ±7.1
Weight (kg)	50.6 ±8.4	49.6 ±6.2	48.3 ±3.0*	54.3 ±9.8	54.9 ±8.8	56.5 ±7.9*
BMI (kg/m²)	21.1 ±2.3	21.1 ±2.0	21.0 ±2.0*	22.7 ±3.7	22.4 ±3.4	23.3 ±2.5*
Parity (n)	1.8 ±1.7	2.4 ±1.7	1.8 ±1.1*	3.0 ±2.6	2.3 ±1.7	3.6 ±2.0*
Fish consumption (times/week)	2.9 ±1.0	3.3 ±1.7	2.7 ±1.9	4.0 ±2.0	3.5 ±2.0	3.0 ±2.1
Infant						
Gestational age at delivery (weeks)	32.9 ±2.4*	32.3 ±2.6*	32.2 ±2.1*	39.2 ±1.4*	40.0 ±2.0*	38.2 ±0.8*
Gender (%male)	43	39	20	46	25	n.e.[c]
Birth weight (kg)	1.7 ±0.3*	1.5 ±0.3*	1.5 ±0.4*	3.0 ±0.5*	3.1 ±0.4*	2.9 ±0.2*
Age at milk sampling (days)	3.5 ±1.0	10.1 ±2	24.5 ±11.5§	3.1 ±0.5§	10.6 ±2	27.3 ±8.2§

[a], preterm and term counterparts with a common symbol (*) differ, $p<0.05$
[b], colostrum and mature counterparts with a common symbol (§) differ, $p<0.05$
[c], not estimated

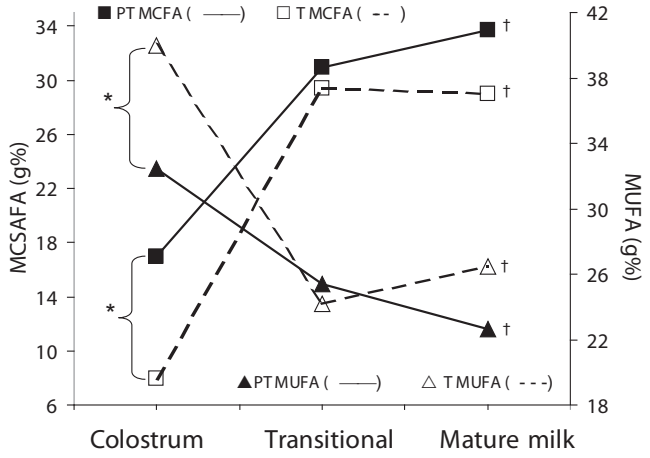

Figure 1. The courses of MUFA and MCSAFA from colostrum to transitional and mature milks in Sengerema (Tanzania) after preterm and term delivery. Data are in g/100 g fatty acids (g%). Preterm delivery (——, closed figures, PT), and term delivery (- - -, open figures, T). MUFA, mono-unsaturated fatty acids; MCSAFA, medium chain saturated fatty acid (i.e. ≤14C-atoms); Colostrum, 2-5 days; Transitional milk, 6-15 days; Mature milk,16-56 days; preterm delivery, 28-36 weeks; term delivery, 37-42 weeks. Statistics: *, significant difference between preterm and term milk at $p<0.05$; †, significant difference between colostrum and mature milk at $p<0.05$.

Table 2. Preterm and term colostrum. transitional and mature milk fatty acid compositions and the direction of change from colostrum to mature milk[a,b]

	Preterm milk			Term milk			Direction of change[c]				
Milk (n)	*Colostrum (14)*	*Transitional (23)*	*Mature (13)*	*Colostrum (30)*	*Transitional (19)*	*Mature (34)*	PT vs. T[d]			M vs. C[e]	
	median(range)	median(range)	median(range)	median(range)	median(range)	median(range)	*C*	*T*	*M*	PT	T
	milk fatty acid composition in g/100 g fatty acid (g%)										
SAFA											
6:0	0.10(0.08-0.17)	0.14(0.06-0.28)	0.14(0.08-0.21)	0.11(0.07-0.15)***	0.15(0.08-0.22)	0.15(0.12-0.25)***.§§§					↑
8:0	0.13(0.05-0.42)***.§	0.43(0.11-0.81)	0.61(0.32-0.65)***	0.06(0.03-0.30)***.§	0.48(0.16-0.67)	0.47(0.28-0.88)***	↑			↑	↑
10:0	0.99(0.22-2.69)***	2.55(0.87-3.84)	2.96(2.14-4.02)***	0.42(0.22-1.86)***	2.79(0.97-3.52)	2.69(1.84-5.15)***				↑	↑
12:0	6.29(1.61-12.4)***.§	12.9(5.48-15.9)	14.2(8.07-19.1)***	2.48(1.36-9.92)***.§	13.1(4.41-16.2)	12.7(7.74-23.5)***	↑			↑	↑
14:0	9.27(4.88-15.8)**.§§§	14.2(6.91-17.7)	15.0(6.77-23.2)**	4.62(2.81-13.2)***.§§§	13.0(5.21-20.4)	13.7(7.16-27.4)***	↑			↑	↑
MCSAFA (≤14:0)	16.9(6.92-29.8)***.§§	30.9(14.8-36.4)	33.7(17.4-44.7)***	7.92(4.71-25.4)***.§§	29.4(10.8-39.9)	29.0(10.3-29.7)***	↑			↑	↑
16:0	26.4(22.5-28.2)**	22.7(18.6-28.1)	21.2(18.4-25.1)**	27.0(20.9-33.4)***	21.2(17.7-25.7)	19.6(14.0-26.8)***				↓	↓
18:0	4.55(4.00-6.96)	4.10(3.19-6.92)	4.40(3.12-5.49)	5.27(3.43-7.58)***	4.18(3.48-5.70)	4.20(1.66-5.76)***					↓
20:0	0.19(0.14-0.33)	0.15(0.12-0.22)	0.17(0.11-0.36)	0.20(0.11-0.42)	0.17(0.11-0.27)	0.19(0.05-0.58)					
22:0	0.15(0.08-0.26)	0.12(0.09-0.16)	0.14(0.09-0.31)	0.16(0.10-0.47)	0.15(0.08-0.23)	0.18(0.07-0.47)					
24:0	0.28(0.19-0.41)	0.20(0.11-0.26)	0.20(0.11-0.39)	0.29(0.18-0.69)***	0.19(0.12-0.37)	0.18(0.10-0.40)***				↓	↓
LCSAFA (>14:0)	32.3(27.3-36.9)**	27.6(22.3-35.5)	25.5(23.7-30.6)**	32.8(27.1-39.9)***	26.6(22.6-30.0)	24.3(19.2-31.9)***				↓	↓
SAFA	45.8(40.2-58.4)***.§§	58.1(44.4-62.9)	59.2(42.9-70.0)***	41.9(35.5-54.7)***.§§	55.2(40.2-67.4)	53.4(39.4-76.4)***	↑			↑	↑
MUFA											
14:1ω5	0.09(0.03-0.14)	0.08(0.03-0.15)	0.08(0.05-0.14)	0.11(0.04-0.26)*	0.09(0.04-0.23)	0.08(0.01-0.23)*					↓
16:1ω7	2.65(1.24-4.07)§	2.29(1.14-3.86)	2.01(1.06-2.83)	3.57(0.90-7.69)***.§	1.88(1.02-5.33)	1.74(0.32-4.40)***	↓				↓
18:1ω7	2.44(1.47-3.42)**.§	1.76(1.13-18.9)	1.56(1.04-2.27)**.§	3.05(1.35-5.37)***.§	1.55(0.79-3.55)	1.20(0.62-2.43)***.§	↓		↑	↓	↓
ω7	5.08(2.79-7.39)*.§	4.03(2.35-21.5)	3.27(2.47-5.20)*	7.08(2.31-12.2)***.§	3.39(1.82-8.54)	3.08(0.95-6.77)***	↓			↓	↓
18:1ω9	25.3(19.3-29.9)**.§§§	20.2(14.6-27.5)	18.6(11.8-29.6)**	31.0(16.7-35.3)***.§§§	20.9(15.1-35.7)	22.6(11.2-32.6)***	↓			↓	↓
20:1ω9	0.46(0.29-0.74)***.§§	0.30(0.16-0.53)	0.26(0.14-0.50)***	0.64(0.27-1.10)***.§§	0.34(0.19-0.70)	0.31(0.02-0.59)***	↓			↓	↓
22:1ω9	0.11(0.06-0.20)***	0.07(0.00-0.12)	0.06(0.01-0.09)***	0.14(0.06-0.29)***	0.07(0.02-0.16)	0.06(0.02-0.11)***				↓	↓
ω9	26.2(19.8-31.3)**.§§§	20.8(14.9-28.3)	19.3(12.1-30.2)**	32.0(17.2-36.4)***.§§§	21.5(15.9-37.0)	23.2(11.5-32.8)***	↓			↓	↓
MUFA	32.5(24.9-35.2)***.§§§	25.4(17.8-41.1)	22.6(16.6-34.4)***	40.0(19.9-46.5)***.§§§	24.2(18.4-45.7)	26.5(14.4-35.3)***	↓			↓	↓

PUFA											
18:3ω3	0.41(0.30-0.87)	0.42(0.24-1.12)	0.47(0.32-0.73)	0.47(0.27-1.04)	0.49(0.28-1.34)	0.52(0.21-1.26)					
20:5ω3	0.09(0.01-0.19)	0.07(0.01-0.27)	0.07(0.01-0.44)	0.06(0.01-0.24)	0.05(0.01-0.19)	0.09(0.02-0.29)					
22:5ω3	0.42(0.15-1.25)	0.24(0.06-0.73)	0.24(0.06-0.83)	0.35(0.13-0.98)***	0.17(0.05-0.52)	0.19(0.06-0.50)***					↓
22:6ω3	1.11(0.38-2.03)[§]	0.85(0.19-1.62)[§]	0.75(0.12-2.12)	0.81(0.32-1.43)**.[§]	0.48(0.28-1.18)[§]	0.53(0.24-1.83)**	↑	↑			↓
LCPω3	1.57(0.54-3.47)	1.23(0.27-2.47)	1.07(0.20-3.39)	1.25(0.52-2.45)***	0.69(0.37-1.89)	0.82(0.31-2.63)***					↓
ω3	1.96(1.08-3.91)	1.64(0.87-3.16)	1.50(0.67-4.07)	1.77(0.79-3.13)*	1.35(0.79-2.37)	1.34(0.60-3.04)*					↓
18:2ω6	14.6(8.24-22.3)	12.7(6.02-19.3)	12.1(8.23-20.0)	13.6(8.72-23.1)	13.0(8.59-22.0)	15.8(6.50-24.5)					
20:2ω6	0.86(0.50-1.21)***	0.56(0.25-0.99)	0.53(0.27-0.64)***	0.93(0.43-2.10)***	0.50(0.27-0.91)	0.45(0.23-0.70)***				↓	↓
20:3ω6	0.73(0.52-1.19)	0.56(0.30-0.92)	0.61(0.41-0.98)	0.61(0.35-1.16)	0.54(0.27-1.06)	0.51(0.36-0.94)					
20:4ω6	0.93(0.58-1.85)*	0.71(0.54-1.08)	0.69(0.40-1.04)*.[§]	1.08(0.64-1.94)***	0.67(0.37-0.99)	0.55(0.38-0.85)***.[§]			↑	↓	↓
22:4ω6	0.37(0.17-0.94)***	0.20(0.15-0.41)	0.19(0.11-0.37)***.[§§§]	0.44(0.18-0.89)***	0.19(0.09-0.56)	0.13(0.07-0.17)***.[§§§]			↑	↓	↓
22:5ω6	0.14(0.07-0.30)**	0.10(0.06-0.19)	0.10(0.07-0.22)**	0.12(0.07-0.21)***	0.08(0.04-0.22)	0.08(0.04-0.18)***				↓	↓
LCPω6	3.00(2.14-5.49)***	2.08(1.50-2.80)	2.14(1.70-2.90)***.[§§]	3.25(1.99-5.02)***	2.16(1.32-3.31)	1.76(1.27-2.29)***.[§§]			↑	↓	↓
ω6	17.8(10.6-25.5)	15.3(7.63-21.8)	14.8(10.1-22.1)	17.3(11.4-25.9)	15.2(10.0-24.6)	18.1(7.81-26.3)					
PUFA	20.2(12.6-27.5)*	16.7(9.04-24.7)	16.3(13.3-23.4)*	19.2(12.9-27.5)	16.7(11.4-26.1)	19.7(9.24-26.9)				↓	

[a]. medians for colostrum and mature milk counterparts with a common symbol (*) differ. at *. $p<0.05$; **. $p<0.01$; ***. $p<0.001$

[b]. medians for preterm and term counterparts with a common symbol ([§]) differ. at [§]. $p<0.05$; [§§]. $p<0.01$; [§§§]. $p<0.001$

[c]. first row: PT. preterm; T. term; M. mature; C. colostrum; second row: C. colostrum; T. transitional; M. mature; PT. preterm; T. term

[d]. directions of change indicate ↑/↓. higher/lower content in preterm compared to term colostrum/transitional/mature milk

[e]. directions of change indicate ↑/↓. higher/lower content in mature compared to colostrum preterm/term milk

PUFA. **Figure 2** shows the apparent courses of AA and DHA for both preterm and term deliveries from colostrum to transitional to mature milk. Preterm milk contained higher DHA in colostrum and transitional milk, and higher AA, 22:4ω6 and LCPω6 in mature milk as compared to the corresponding term milks.

Differences from colostrum to mature milk

SAFA and MUFA. Independent of prematurity, milk 6:0, 8:0, 10:0, 12:0, 14:0, MCSAFA (Figure 1) and SAFA increased consistently, while 16:0 and 24:0 decreased. Only term milk showed a decrease in 18:0. Also, 18:1ω7, ω7, 18:1ω9, 20:1ω9, 22:1ω9, ω9 and MUFA (Figure 1) decreased with advancing lactation. Only term milk showed a decrease in 14:1ω5 and 16:1ω7.

PUFA. We noted decreases of 20:2ω6, AA (Figure 2), 22:4ω6, 22:5ω6 and LCPω6. Only term milk showed a decrease in 22:5ω3, DHA (Figure 2), LCPω3 and ω3.

Milk fatty acid interrelationships.

There was a strong inverse relation (**Figure 3**) between MUFA and MCSAFA in the combined milk samples of preterm and term delivering women.

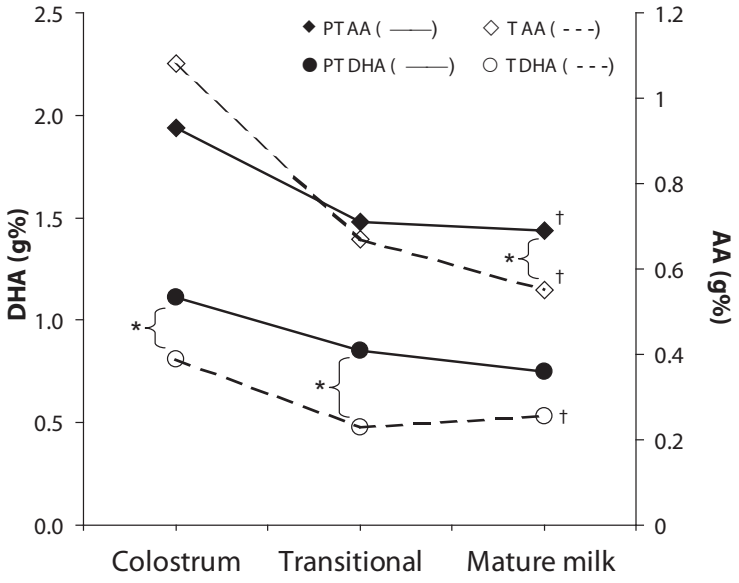

Figure 2. The courses of AA and DHA from colostrum to transitional and mature milks in Sengerema (Tanzania) after preterm and term delivery. Data are in g/100 g fatty acids (g%). Preterm delivery (——, closed figures, PT), and term delivery (- - -, open figures, T). AA, arachidonic acid; DHA docosahexaenoic acid; for other abbreviations and symbols see legend of Figure 1. Statistics: *, significant difference between preterm and term milk at $p<0.05$; †, significant difference between colostrum and mature milk at $p<0.05$.

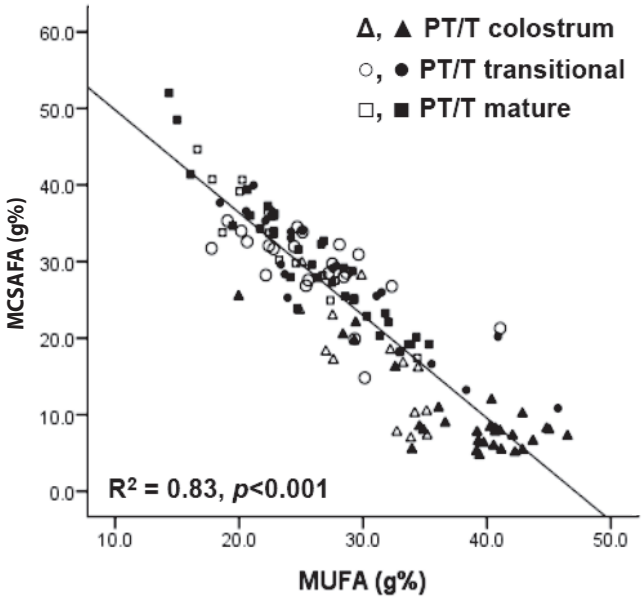

Figure 3. The relation between MCSAFA and MUFA in preterm and term milks in Sengerema (Tanzania) after preterm and term delivery. Preterm milk, open symbols; term milk, closed symbols; R^2, correlation coefficient; *p*, significance; for other abbreviations and symbols see legend of Figure 1.

DISCUSSION

We investigated the FA compositions of preterm and term milks from mothers with lifelong high intakes of tropical fresh water fish in Tanzania. Most importantly, we found higher DHA and MCSAFA, but lower MUFA in preterm colostrum, compared to term colostrum. Differences between preterm and term colostrum vanished with advancing lactation, during which, in both preterm and term milks, MCSAFA increased, while AA, DHA and MUFA decreased (Figures 1 and 2).

Comparison of current FA data with the literature

The compositions of preterm and term colostrum, transitional and mature milks have been described for North,[115] South,[117,125] East[123] and West European countries,[112,120] two South American[124,125] and one Asian country,[126] but no data are available for sub-Saharan African countries. Also, none of these studies focused on a population with high fish intakes. A high DHA, EPA and AA status might be part of human physiology, since there is substantial evidence that *Homo sapiens* evolved in an East African water-land ecosystem with abundant sources of LCP.[16,100,133,134] The high fish intakes of the current Tanzanian population became confirmed by their high milk AA (overall median 0.71 g%) and DHA (0.70 g%) contents.

MCSAFA and MUFA

The difference between the MCSAFA and MUFA content of African compared to European or Western milks, as noted earlier,[135] is remarkable. African preterm and term colostrum, but notably mature milks, have considerable higher MCSAFA contents (16.9 and 7.92 g% MCSAFA in colostrum, respectively; 33.7 and 29.0 g% MCSAFA in mature), compared to counterpart Western preterm and term milks (12 and 10 g% MCSAFA in colostrum, respectively; 15 and 13 g% MCSAFA in mature milks).[112] High milk MCSAFA contents have been related to high carbohydrate intakes[26,108], while the increasing milk MCSAFA content with advancing gestation relates to increasing mammary gland MCSAFA synthetic activity.[105,128] Except for in term colostrum, African milks also show lower MUFA in preterm and term milks (32.5 and 40.0 g% MUFA in colostrum, respectively; 22.6 and 26.5 g% MUFA in mature milks), compared to European preterm and term milks (35 and 36 g% MUFA in both colostrum and mature).[122] Both, a higher milk MCSAFA and lower milk MUFA content was noted earlier in rural South African[136] and rural Tanzanian women.[111] Although mostly attributed to their high carbohydrate intakes, we recently suggested,[137] that these differences might in part also be secondary to a higher insulin sensitivity is these rural women, resulting in lower milk MUFA and 16:0 from insulin induced adipolysis, hepatic VLDL- and *de novo* lipid synthesis and lower mammary gland LPL-activity.

DHA and AA

We compared our data with worldwide data from other studies using capillary gas chromatography columns. We excluded data that derived from analyses with a packed gas chromatography columns since the use of such columns might lead to artificially high values.[98] The current median DHA content of 1.11 in preterm colostrum is higher compared to the 0.30-0.66 g% DHA range as previously established for preterm colostrum by others.[112,115,117,120,123,125,126] This value is consequently the highest reported in the literature so far by lack of data from prematurely delivering women with high fish intakes. The median DHA content of 0.81 g% in term colostrum is, however, within the 0.13-1.10 g% DHA range, as previously established for term colostrum,[115-117,119,120,123,126] for review up to 2000 see.[138] The extreme value of 1.10 g% derived from our earlier study in women living on the Caribbean island of St. Lucia, who also have regular high DHA intakes from local fish.[139]

The median AA contents of 0.93 g% in preterm and 1.08 g% in term colostrum are within the reported range of 0.59-1.13 g% for preterm colostrum [[117,126], for review up to 2007,[122]] and the 0.52-1.60 g% range for term colostrum [[115-117,120,123,126], for review up to 2000 see[138]]. Apart from dietary differences, the wide range in reported values might to some extent also derive from the lack of uniformity in the reported FA. For instance, the short-chained 6:0 and 8:0, the very long chain 24:0 and 26:0, the uneven carbon chain numbered 17:0 and/or 19:0 or the unsaturated 14:1ω5, 16:1ω9, 24:1ω9 or 20:3ω3 are not always included in the cited references.

The median 0.75 g% DHA in preterm mature milk in the current study is higher compared to the so far reported 0.19-0.39 g% DHA range in preterm mature milk,[117,120,122,124] but the 0.53 g% DHA in

term mature milk is within the previously reported range of 0.06-1.79 g% DHA.[111,119] The median 0.69 g% AA in preterm mature milk in the current study is somewhat higher compared to the previously reported 0.36-0.59 g% AA range in premature mature milks,[115,123] while the 0.55 g% AA in term mature milk is within the reported range of 0.26-1.00 g% AA in mature term milks.[8,115,123,136,140] Finally, the observation that both AA and DHA are relatively high in milk from women with high fish intakes is in line with data from term delivering women with high intakes of African freshwater fish[59] and counterparts in St. Lucia with frequent saltwater fish intakes.[139]

Changes of DHA and AA with advancing lactation

The decreases of both DHA and AA in milk with advancing lactation is well documented[112,113,115,119,122,123] It is usually explained by the increasing fat output[121,141] and fat globule size[142-144] with advancing lactation, which causes a decrease of the phospholipid/triglyceride ratio.[142] The latter coincides with a decreasing contribution of LCP to the total milk FA content, since LCP have a preference for incorporation into phospholipids.[114,145] The question 'why lipid droplets increase in size' has, however, not been explained clearly as yet. It might simply be secondary to the maturation of the mammary gland,[105,142] but ultimately also be the consequence of the depletion of maternal LCP stores, necessitating a shift from small lipid droplets (with relatively high phospholipid content) towards large counterparts (with relatively low phospholipid content). The latter option seems less likely, in view of the high AA and DHA status of the current women.

We[146] recently suggested that the rapidly changing hormonal milieu after delivery, notably the restoring insulin sensitivity, might influence various enzymatic activities such as those of the FA-desaturases (FADS), those involved in hepatic *de novo* fatty acid synthesis and in adipolysis. We hypothesized that the observed postpartum changes in maternal and infant RBC-FA-compositions might be the result of these changing enzymatic activities, secondary to this restoring insulin sensitivity. Interestingly, the present results support this previous hypothesis, but this would need confirmation from longitudinal data and a concurrent estimation of maternal insulin sensitivity. However, if MUFA are considered as a proxy for hepatic *de novo* lipogenesis and adipolysys, as we suggested earlier,[146] these preliminary data suggest that with advancing lactation, *de novo* synthetic activity in the mammary gland replaces in part those FA derived from adipose tissue lipolysis and those from *de novo* synthetic activity in the liver,[146] which might be a remnant of the insulin resistance of late pregnancy.[137]

Fatty acid differences between preterm and term milks

Differences between preterm and term human milks do not seem to have a molecular basis, since no differences in triglyceride-structure,[147] phospholipid-classes,[145] percentages phospholipids from total lipid,[148] average fat globule diameter or fat globule surface area[144] have been reported. We have previously shown that pregnant women in the currently investigated population exhibit no changes in RBC-DHA during pregnancy[131] and have higher RBC-DHA than their infants at delivery.[130] The

maternal RBC-DHA content decreases slightly during subsequent lactation, while the infant RBC-DHA increases rapidly to reach maternal levels within 3 months.[130] This suggests that the lifetime high fish intake in these women has caused a state of DHA equilibrium during pregnancy and to a lesser extent during lactation. Consequently, maternal DHA depletion in the period between preterm and term birth is unlikely to be the cause of the presently observed higher DHA in preterm colostrum compared with term colostrum or the only cause of the similar preterm-term colostrum DHA differences that have consistently been documented in the literature.

Alternatively, differences in insulin sensitivity at the time of delivery might play a role in the observed FA differences between preterm and term colostrum,[137] since in premature delivery pregnancy is terminated at a stage of higher insulin sensitivity compared with termination at term. Consequently, lower MUFA in preterm colostrum, might also derive from lower insulin induced adipolysis and *de novo* FA synthesis in the maternal liver. Thus, in contrast to Freed et al.,[128] who suggested that the higher MCSAFA in preterm milk derives from higher *de novo* FA synthesis in the preterm mammary gland, we rather suggest that it derives from lower uptake of FA from adipose tissue lipolysis and lipogenesis in the preterm maternal liver. Rerouting of lipid and glucose fluxes after delivery becomes supported by the post delivery upregulation of the number of insulin receptors[149] and of lipoprotein lipase activity in the mammalian mammary gland[149,150] and their concomitant downregulation in the liver and adipose tissue.[149]

Strengths and limitations

A strength of this study is the uniform lifetime consumption of a diet with very little intra- and inter-individual variation. We did not investigate maternal RBC or circulating lipids in this particular study and can therefore not irrefutably exclude some degree of DHA depletion in the currently studied women during pregnancy. The notion of no depletion rests on a concurrent study of women in the same hospital who were investigated during pregnancy, at delivery and at 3 months postpartum, and of which the data showed no DHA depletion as based on RBC-DHA data during pregnancy.[130,131] Another limitation is the current cross-sectional design. More statistical power would have been obtained from truly longitudinal data. We experienced, however, that in the encountered setting a longitudinal study is almost impossible for both ethical and logistical reasons since the included African women have more important tasks (multiple children to feed) than regularly (notably punctually) attending a hospital. Some of them are nomadic and do not ever attend the same hospital, while very few see the purpose of participation.

CONCLUSIONS

The high lifelong fish intakes by these African women living on the shore of Lake Victoria has provided us with data on milk AA and DHA that are the highest reported for colostrum (DHA) and mature preterm (DHA and AA) milks so far. Consistent with the literature, African milks exhibit a substantially higher MCSAFA, and lower MUFA contents, compared to Western milks. Similarly,

preterm colostrum contains higher DHA and MCSAFA and lower MUFA compared to term colostrum. Each of these differences vanishes rapidly with advancing lactation, during which the LCP and MUFA decreased and MCSAFA increased both in preterm and term milks. The high DHA status of these lactating women suggests that the encountered DHA differences between preterm and term colostrum are unlikely to originate from maternal DHA depletion taking place in the relatively short period between preterm and term delivery. Conversely, the consistently found higher MCSAFA and lower MUFA in preterm compared to term colostrum in the present study and the literature might derive from insulin-sensitivity driven differences in maternal lipid and glucose fluxes at the time of delivery and insulin-sensitivity driven changes with advancing lactation, but these speculations clearly need testing by appropriate measurements of insulin sensitivity and the assays of adipose tissue lipolytic activities and *de novo* FA synthetic activities of both the liver and breast by stable isotope techniques.

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CHAPTER 4.1

A maternal erythrocyte DHA content of approximately 6 g% is the DHA status at which intrauterine DHA biomagnification turns into bioattenuation and postnatal infant DHA equilibrium is reached

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ABSTRACT

Introduction. Higher LCP in infant compared with maternal lipids at delivery is named biomagnification. The decline of infant and maternal DHA status during lactation in Western countries suggests maternal depletion. We investigated whether biomagnification persists at lifelong high fish intakes and whether it prevents a postpartum decline of infant and/or maternal DHA status.

Methods. We studied 3 Tanzanian tribes with low (Maasai; 0/week), intermediate (Pare; 2-3/week) and high (Sengerema; 4-5/week) fish intakes. DHA and AA were determined in maternal (m) and infant (i) erythrocytes (RBC) during pregnancy (1st trimester n=14; 2nd=103; 3rd=88), and in mother-infant pairs at delivery (n=63) and at 3 months postpartum (n=104).

Results. At delivery, infants of all tribes had similar iRBC-AA, which was higher than, and unrelated to, mRBC-AA. Transplacental DHA biomagnification occurred up to 5.6 g% mRBC-DHA; higher mRBC-DHA was associated with 'bioattenuation' (i.e. iRBC-DHA< mRBC-DHA). Compared to delivery, mRBC-AA after 3 months was higher, while iRBC-AA was lower. mRBC-DHA after 3 months was lower, while iRBC-DHA was lower (Maasai), equal (Pare) and higher (Sengerema) compared to delivery. We estimated that postpartum iRBC-DHA-equilibrium is reached at 5.9 g%, which corresponds to a mRBC-DHA of 6.1 g% throughout pregnancy.

Conclusion. Uniform high iRBC-AA at delivery might indicate the importance of intrauterine infant AA-status. Biomagnification reflects low maternal DHA-status and bioattenuation may prevent intrauterine competition of DHA with AA. A mRBC-DHA of 6 g% during pregnancy predicts maternal-fetal equilibrium at delivery, postnatal iRBC-DHA equilibrium, but is unable to prevent a postnatal mRBC-DHA decline.

INTRODUCTION

The long-chain polyunsaturated fatty acids (LCP) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) are structural components of membrane phospholipids.[1] DHA and AA are notably abundant in the central nervous system and play important roles in fetal and infant neurodevelopment.[2] DHA, EPA and AA are derived either from the diet or from endogenous synthesis from their respective parent essential fatty acids (EFA) linoleic acid (LA) and alpha-linolenic acid (ALA). Fatty fish are rich sources of EPA and DHA.. Dietary sources of preformed AA are meat, eggs and poultry,[3] while lean and tropical fish are rich sources of AA.[4] It is suggested that LCP intakes from our Paleolithic diet have been much higher[5-7] than presently observed in the majority of affluent countries.[8,9] The high AA and DHA contents of our Paleolithic diet may imply that human evolution, and thus also intrauterine development, took place at high maternal intakes of both AA and DHA.[7]

Pregnant and lactating women have high LCP needs.[10] This is notably the case for DHA, which becomes synthesized from ALA with difficulty.[11] Both AA and DHA are important during infant development. More AA than DHA accretes in brain in the intrauterine period, while DHA accretes rapidly from week 30 of pregnancy to 2 years postpartum.[12] The commonly observed higher LCP in circulating cord plasma lipids as compared to maternal plasma lipids has been coined 'biomagnification'.[13] The decline of infant DHA status during lactation and of the maternal DHA status during pregnancy[14] and lactation[15] might reflect the low LCPω3 status in many Western countries.

The outcome of randomized controlled trials aiming at improvement of neurodevelopment by supplementation of pregnant women or their infants with fish oil, DHA,[16-18] or DHA+AA,[19,20] indicate subtle positive effects at most. This contrast with the positive relations between neonatal brain DHA and cognitive and behavioral performance that are noted in the combined human and animal studies.[21,22] Failure to show favorable effects of LCP might, among many other reasons, relate to the use of too low dosages and the study of Western populations with low fish intakes without stratification based on baseline DHA status.[6]

There is substantial evidence that land-water ecosystems have been exploited by our early African ancestors[7,23-26] and it is also known that some rural African populations have much higher milk LCP contents[4,27] as compared to those living in Western countries. Data on the concurrent maternal and infant LCP status during pregnancy and lactation in these African populations are however currently lacking.

In view of this lack of data, we determined the maternal and infant DHA and AA status in 3 traditional rural African populations with different levels of fish intakes. Since a longitudinal study design proved impossible in these populations, we collected data deriving from different women during pregnancy, at delivery and at 3 months postpartum. The selected populations live in Tanzania and have low (Maasai), intermediate (Pare), or high (Sengerema) intakes of freshwater fish. All 3 populations are characterized by the consumption of diets that are constrained in their varieties by the availability of the food in their immediate environment. Red blood cell (RBC) LCP contents

served as proxies for LCP status. We were particularly interested to see whether 'biomagnification' and maternal DHA losses during pregnancy and lactation also occur at lifetime high intakes of fish and at what maternal LCP status the infant reaches a state of postnatal LCP equilibrium.

SUBJECTS AND METHODS

Subjects, diet and cultural circumstances

We selected 3 Tanzanian ethnic tribes with different intakes of local freshwater fish, i.e. the Maasai (no or low fish intake, 0/wk), subjects from the Pare Mountains (intermediate fish intake, 2-3/wk) and subjects from Sengerema (high fish intake, 4-5/wk). We[4] have previously shown that East African fresh water fish contain high proportions of both DHA and AA and relatively low EPA as compared to European salt water fish. We experienced that collection of longitudinal data was virtually impossible. Appointments were not scheduled, nor were we able to motivate subjects to participate in a longitudinal study.

The selected Maasai were Nilotic pastoralists who live in 'bomas' (villages) in the Maasai Steppe nearby Ruvu. Their diet consists mainly of curdled milk and meat. It has recently become replenished with some ugali (corn porridge). Consumption of fish is uncommon, since it is considered inedible. The Pare group was composed of women of the Bantu tribes Pare and Sambaa from the Pare Mountains. Their diet was mainly composed of vegetables, beans and fruits with ample ugali, rice and chapati (cornwheat pancakes), and some meat or fish. The third population was composed of women from Sengerema (southern shore of Lake Victoria). Apart from the abundant consumption of fish, they also consume ugali, muhogo (cassava root) and plantain (baked banana).

The ethnicity/tribe of each of the study groups was considered to be homogeneous. Most women were of low socio-economical background. Their incomes were derived from pastoralism (Maasai), agriculture (Pare) or fisheries (Sengerema). The studied populations neither had possibilities nor interest in changing their cultural habits, including their diets. Local hospital staff members and interviews with the participants confirmed that neither pregnancy nor lactation is associated with any change in dietary habits, or the prohibition of certain foods (F. Peters, R.S. Kuipers, F.A.J. Muskiet, *unpublished data*). Their dietary compositions were therefore likely to be representative for the lifetime dietary habits of each of the ethnical groups.

Women were included if they were apparently healthy and well nourished, and had delivered an apparently healthy child at term (37-42 weeks, by estimate). Use of tobacco and alcohol are almost non-existent in these populations, especially among women. Anthropometric data and a questionnaire on fish intake were obtained from the medical records or by interviews in Kiswahili. Besides the measurement of fundal heights, reliable data on gestational age were not always available, since some women had no recollection of their last menstrual period. Devices for echo imaging were either not available or not operational. All women gave their informed consent. The study was approved by the National Institute for Medical Research in Dar-es-Salaam (NIMR/HQIR.8a/Vol. IX/145, dated June 16, 2003 and NIMR/HQ/R.8a/Vol. IX/800, dated April 8, 2009) and was in

agreement with the Helsinki declaration of 1975 as revised in 2000.

Samples and analyses

We collected about 4 mL EDTA-anticoagulated venous blood of the mothers during pregnancy and at delivery and about 4 mL EDTA-cord blood at delivery (BD Vacutainer, Plymouth, UK). At 3 months postpartum, about 4 mL EDTA-anticoagulated venous blood of the mothers was collected. About 250 µL EDTA-anticoagulated blood (250 µL pediatric MiniCollect K_3EDTA-tubes; Greiner Bio-one, Kremsmünster, Austria) was taken by heel prick from the 3 months old infants. The samples were stored at 4°C in the dark and processed within 2 h after collection. RBC were isolated by centrifugation and washed three times with 0.9% NaCl. After washing, 200 uL of the RBC suspension (mothers) or the entire RBC suspension (infants) was transferred to a teflon-sealable Sovirel tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and 50 µg 17:0. In this ready-to-transmethylate mixture fatty acids are stable at room temperature and in the dark for months. All samples were transported at ambient temperature to the University Medical Center Groningen (The Netherlands) for fatty acid analysis. Within 9 months after collection, the methanol-HCL conserved samples were transmethylated by heating at 90 °C for 4 h. Subsequent analysis of fatty acid methyl esters was performed, by capillary gas chromatography/flame ionization detection according to previously described procedures.[28] Fatty acid compositions and their ratios were expressed in g% and g/g, respectively.

Statistics

Statistical analyses were performed with SPSS version 16.0 (SPSS Inc, Chicago, IL). Within-tribe differences of mothers and infants at delivery were compared with corresponding data at three months postpartum by use of the Mann-Whitney U-test. Mother-infant pair differences were tested with a Wilcoxon two related samples test. In both instances $p<0.05$ was considered significant. Between-group differences were studied with the aid of the Kruskal-Wallis test, followed by analyses with Mann Whitney U-test (non-parametric) at $p<0.05$. Corrections were made for type-1 errors (Bonferroni correction). Equations were derived from linear regression analysis. We used the coefficient of determination (R^2) to estimate the extent to which a given variable was explained by another.

RESULTS

Study groups

Pregnant women

We included 205 different pregnant women. Of these, 14 women were included in their 1st trimester of pregnancy (defined as 1 -13 weeks; 3 Maasai, 4 Pare, 7 Sengerema), 103 women in the 2nd trimester (14-27 weeks; 14 Maasai, 47 Pare, 42 Sengerema) and 88 women in the 3rd trimester (28 weeks-delivery; 16 Maasai, 41 Pare, 31 Sengerema). **Table 1** shows the characteristics of all pregnant

Table 1: Characteristics of pregnant women and mother-infant couples at delivery and at 3 months PP

	Maasai	*Pare*	*Sengerema*
Pregnant women			
Maternal age, *y*	25 ± 8 (33)	26 ± 6 (92)	26 ± 6 (80)
Weight, *kg* trimester 1	50.7 ± 6.4 (3)	54.8 ± 16.5 (4)	54.9 ± 8.2 (7)
Weight, *kg* trimester 2	52.1 ± 4.4 (14)[a]	56.8 ± 8.2 (47)[b]	58.6 ± 7.0 (42)[b]
Weight, *kg* trimester 3	52.9 ± 4.7 (16)[a]	59.3 ± 8.9 (41)[b]	61.5 ± 3.9 (31)[b]
Height, *m*	1.60 ± 0.06 (33)	1.57 ± 0.10 (92)	1.59 ± 0.06 (80)
BMI, *kg/m²*	20.8 ± 1.4 (33)[a]	23.6 ± 3.2 (92)[b]	23.6 ± 2.7 (80)[b]
Gravida, *n*	4 ± 3 (32)[b]	3 ± 1 (92)[a]	4 ± 2 (80)[b]
Para, *n*	3 ± 3 (32)[b]	1 ± 1 (92)[a]	3 ± 2 (79)[b]
Fish intake, *times/wk*	0 ± 0 (33)[a]	2 ± 1 (92)[b]	4 ± 2 (70)[c]
Delivery			
Maternal age, *y*	23 ± 4 (8)	25 ± 7 (31)	24 ± 7 (34)
PP weight, *kg*	53.0 ± 5.0 (8)	57.3 ± 7.6 (23)	54.4 ±10.2 (31)
Height, *m*	1.59 ± 0.06 (8)[a]	1.54 ± 0.04 (27)[b]	1.56 ±0.06 (31)[a,b]
BMI, kg/m²	20.9 ±2.0 (8)[b]	23.9 ± 2.0 (21)[a]	22.4 ± 3.3 (31)[b]
Gravida, *n*	3 ± 1 (8)	3 ± 2 (31)	3 ± 3 (34)
Para , *n*	2 ± 1 (8)	2 ± 2 (31)	2 ± 3 (34)
Gestational age at birth, *wk*	40.0 ± 0 (1)	39.9 ± 1.5 (17)	38.9 ± 1.8 (28)
Fish intake, *times/wk*	0 ± 0 (8)[a]	3 ± 2 (17)[b]	5 ± 2 (30)[c]
Infant birth weight, *g*	3050 ± 400 (8)	3115 ± 535 (26)	2947 ± 751 (34)
Gender, *% male*	38 (8)	45 (29)	58 (36)
3 months PP			
Maternal age, *y*	22 ± 4 (9)	24 ± 4 (40)	24 ± 6 (61)
PP weight, *kg*	52.4 ± 3 (9)	52.2 ±11 (40)	54.6 ± 10 (61)
Height, *m*	1.60 ± 0 (9)	1.55 ± 0.07 (40)	1.56 ± 0.06 (61)
BMI, kg/m²	20.5 ± 1.1 (9)	21.6 ± 4.0 (40)	22.1 ± 3.0 (61)
Para, *n*	3 ± 1 (9)	2 ± 1 (40)	3 ± 2 (61)
Fish intake, *times/wk*	0 ± 0.7 (8)[a]	3 ± 2 (40)[b]	4 ± 2 (61)[c]
Infant age, *wk*	16 ± 4 (9)	14 ± 3 (40)	13 ± 2 (61)
Gender, *% male*	67 (9)	47 (40)	55 (61)

Values are mean ± SD (n). Different superscripts indicate significant different means, $P < 0.017$.

women. There were no between-trimester differences in the general characteristics for each of the tribes. From the data of different trimesters only body weight is shown. The Pare ($p<0.005$) in the 2nd trimester, and Sengerema ($p<0.001$) women in the 2nd and 3rd trimester, were heavier than the Maasai women and had a higher BMI during pregnancy compared to the Maasai ($p<0.001$). The number of previous pregnancies was lower in Pare women, compared with Maasai ($p=0.003$) and Sengerema ($p=0.003$) women. The, for the current study most relevant between-tribe difference, was in fish intake. Fish consumption occurred in the order Sengerema>Pare>Maasai.

Mother-infant pairs at delivery and at 3 months postpartum

We included 73 mother-infant pairs at delivery (8 Maasai, 31 Pare, 34 Sengerema) and 110 different mother-infant pairs (9 Maasai, 40 Pare, 61 Sengerema) after three months of exclusive breastfeeding. Their characteristics are also shown in Table 1. There were no between-tribe differences for the mothers at delivery or at 3 months postpartum, except for a lower BMI at delivery ($p=0.001$) and higher length ($p=0.015$) of the Maasai mothers compared to Pare mothers. Also in these groups

fish consumption occurred in the order Sengerema>Pare>Maasai at delivery and at 3 months postpartum.

Erythrocyte AA and DHA

RBC-FA data were available from all 205 women during pregnancy. At delivery we studied different subjects. RBCs were available of 6 Maasai, 27 Pare and 34 Sengerema women, and 8 Maasai, 29 Pare and 36 Sengerema infants. At three months postpartum we included a group of different subjects including 9 Maasai, 38 Pare and 60 Sengerema women, and 8 Maasai, 38 Pare and 61 Sengerema infants. Drop-out was due to failure to obtain consent for both mother and infant, early discharge (only at delivery) and logistical and analytical imperfections (delivery and 3 months postpartum).

Between- and within-tribe RBC-AA differences and mother-infant RBC-AA relation

Figure 1A (left panel) shows the apparent maternal and infant RBC-AA courses from the first trimester to 3 months postpartum. For convenience, data points are connected with dotted lines, but it should be noted that they are derived from different study groups. Visual inspection of the apparent courses of maternal RBC-AA during pregnancy did not reveal consistent changes. It seemed to decreased in Pare (intermediate fish), but appeared constant in Maasai (low fish) and Sengerema (high fish) mothers. We analyzed *between-tribe* maternal RBC-AA differences at delivery. RBC-AA of Maasai women was higher than RBC-AA of Pare (p=0.005) and Sengerema (p<0.001),

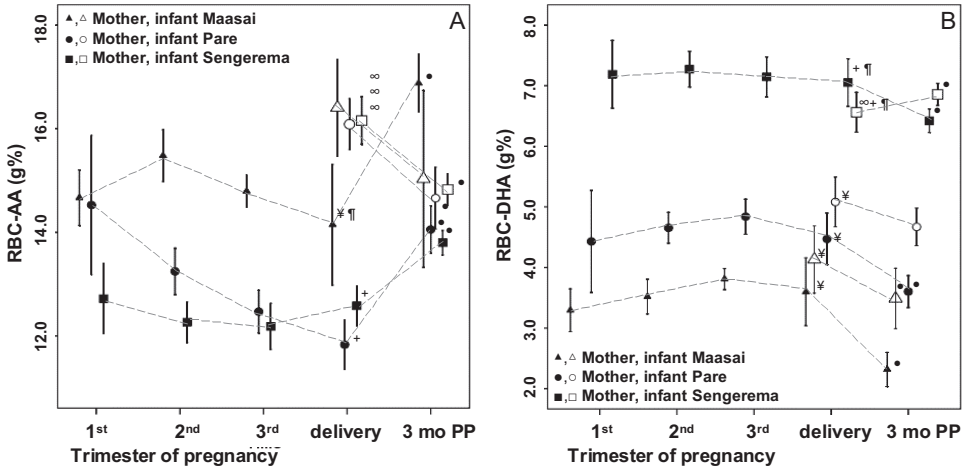

Figure 1. Apparent courses of red blood cell (RBC) arachidonic acid (AA; panel A) and docosahexaenoic acid (DHA, panel B) from the first trimester of pregnancy up to 3 months postpartum for Maasai (low fish), Pare (intermediate fish) and Sengerema (high fish) women and infants. Data represent means±2SEM in g/100 g (g%) fatty acids. Data derive from different maternal subgroups at the 1st, 2nd and 3rd trimester and different mother-infant pairs at delivery and 3 months postpartum. Maternal and infant data are represented by closed and open symbols, respectively. PP, postpartum. + significantly (sign) different from Maasai, ¶ sign different from Pare, ¥ sign different from Sengerema, ● sign different from delivery, ∞ sign different from mother.

whereas Pare and Sengerema women did not show differences. The RBC-AA of the infants showed no between-tribe differences at delivery.

Within-tribe analysis of RBC-AA showed that in Maasai, Pare and Sengerema mothers RBC-AA at delivery was lower than at 3 months postpartum ($p \leq 0.001$). In Maasai infants, there were no differences in RBC-AA between delivery and 3 months postpartum, whereas in Pare and Sengerema infants, RBC-AA was significantly higher at delivery as compared to 3 months postpartum ($p \leq 0.001$). In all tribes, we found that at delivery, infant RBC-AA was significantly higher compared to maternal RBC-AA (Maasai $p=0.028$, Pare $p<0.001$ and Sengerema $p<0.001$), but there was no relation between infant RBC-AA and maternal RBC-AA (**Figure 2A**).

Taken together, maternal RBC-AA was consistently lower at delivery compared to 3 months postpartum. Infant RBC-AA contents were similar at delivery and consistently higher than maternal RBC-AA. Thus, biomagnification of AA during pregnancy occurred, irrespective of maternal AA status (Figures 1A and 2A). After 3 months of exclusive breastfeeding, infant RBC-AA was lower compared to delivery in Pare and Sengerema. This decline did not reach significance for the Maasai, probably because of low numbers.

Between- and within-tribe RBC-DHA differences, and mother-infant RBC-DHA relation

Figure 1B (right panel) shows the apparent courses of maternal and infant RBC-DHA contents from the first trimester to 3 months postpartum. Visual inspection of maternal RBC-DHA during pregnancy revealed a trend of higher RBC-DHA in late pregnancy of the Pare and the Maasai, but not the women in Sengerema. Analysis of the *between-tribe* maternal and infant RBC-DHA differences

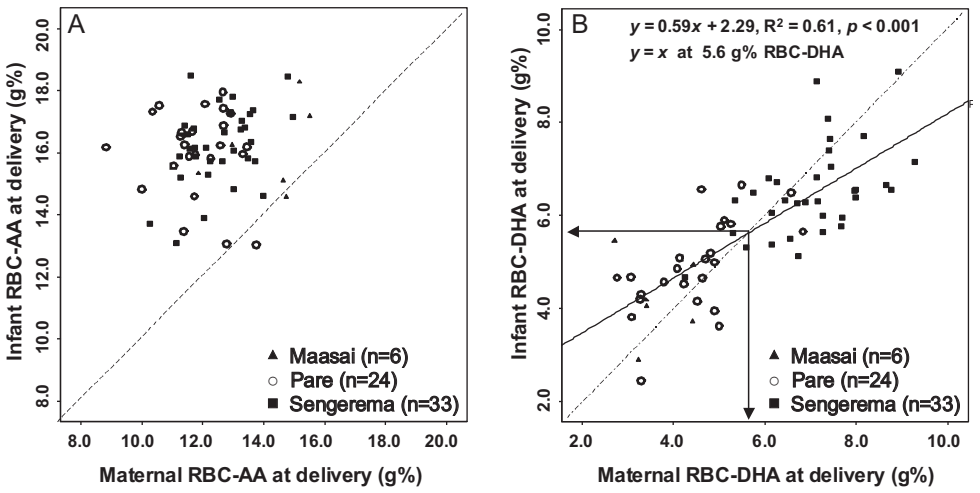

Figure 2. Relations between maternal and infant red blood cell (RBC) contents of AA (panel A) and DHA (panel B) at delivery for Maasai (n=6), Pare (n=24) and the Sengerema (n=33) women and infants. Dotted lines indicate *y*=x for 'equal AA or DHA sharing'. Maternal vs. infant RBC-AA at delivery is insignificant. At delivery, maternal RBC-DHA equals infant RBC-DHA at 5.6 g%.

showed that at delivery, RBC-DHA in Sengerema women was higher compared to Pare and Maasai ($p<0.001$) whereas Pare and Maasai women showed no differences.

Within-tribe RBC-DHA differences showed that in all tribes, maternal DHA at delivery was higher than at 3 months postpartum (Maasai $p=0.003$; Pare and Sengerema $p\leq0.001$). In the infants, Maasai had higher RBC-DHA at delivery compared to 3 months postpartum ($p=0.039$); Pare did not show a significant difference between delivery and 3 months postpartum, while Sengerema infants showed a lower RBC-DHA at delivery compared to 3 months postpartum ($p=0.027$).

At delivery, the analysis of mother-infant-pairs differences revealed that Maasai ($p=0.245$) and Pare ($p=0.091$) infants, although insignificant, tended to have higher RBC-DHA than their mothers, while (in contrast) the RBC-DHA of the Sengerema infants was lower ($p=0.029$) compared to their mothers. The relationship between *maternal and infant RBC-DHA* at delivery is shown in **Figure 2B**. For this analysis we selected data of complete mother-infant pairs (6 Maasai, 24 Pare and 33 Sengerema). At low maternal DHA status, infants usually had higher RBC-DHA than their mothers (biomagnification), but in contrast, at high maternal DHA status, infant RBC-DHA was generally lower ('bioattenuation'). Figure 2B indicates that equal RBC-DHA in mothers and infants at delivery was reached at a maternal RBC-DHA of 5.6 g%.

Although our data are not longitudinal, it appeared that at low maternal RBC-DHA infant RBC-DHA decreased from delivery until 3 months postpartum, whereas at high maternal DHA status, infant RBC-DHA increased from delivery until 3 months postpartum. We therefore calculated the maternal DHA status at which the infant appeared to be in a state of postpartum DHA-equilibrium. In other words, we estimated the maternal RBC-DHA status from which the infant showed no changes in RBC-DHA from delivery to 3 months postpartum.

Figure 3 shows the relation between infant RBC-DHA at delivery and at 3 months postpartum for the Maasai, Pare and Sengerema. Only mean values for each of the tribes could be used, since we did not study the same infants at delivery and at 3 months postpartum. Figure 3 indicates that a postpartum infant RBC-DHA equilibrium was reached at an infant RBC-DHA of 5.9 g% at delivery. This infant RBC-DHA in turn corresponds with a maternal RBC-DHA of 6.1 g% at delivery (by inserting 5.9 into equation of Figure 2B). The number of included Maasai was low. Exclusion of their data did, however not change the equation in Figure 3.

Taken together, maternal RBC-DHA contents at both high and low DHA status were lower after 3 months of exclusive lactation, compared with delivery. Biomagnification of DHA seems to occur up to a maternal RBC-DHA of 5.6 g% at delivery, from this turning point DHA becomes 'bioattenuated'. Postpartum infant RBC-DHA equilibrium was reached at an infant RBC-DHA of about 5.9 g% at delivery, corresponding to a maternal RBC-DHA of 6.1 g% during pregnancy and at delivery. For all practical reasons we conclude that biomagnification takes place up to a maternal RBC-DHA status of about 6 g%, and that also infant postnatal RBC-DHA equilibrium is reached at about 6 g%.

DISCUSSION

We determined the RBC-AA and DHA contents of pregnant Tanzanian women, and of mother-infant pairs at delivery and at 3 months postpartum. The mothers had stable dietary intakes characterized by low (Maasai), intermediate (Pare) and high (Sengerema) consumption of freshwater fish. Our main findings are that biomagnification of AA occurs irrespective of maternal AA status. In contrast to maternal RBC-AA, infant RBC-AA at delivery was remarkably uniform and also after 3 months exclusive lactation, although to a lesser extent. There was no relation between maternal and infant

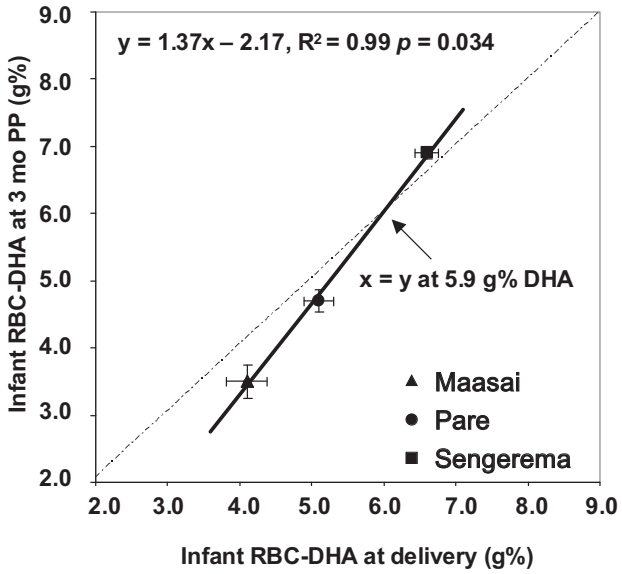

Figure 3. Relation between the mean red blood cell (RBC) docosahexaenoic acid (DHA) at delivery and at 3 months postpartum for the Maasai, Pare and Sengerema infants, respectively. Data represent means±2SEM in g/100 g (g%) fatty acids. Infant RBC-DHA at delivery equals infant RBC-DHA at 3 months postpartum at 5.9 g%.

RBC-AA. From delivery to 3 months postpartum maternal RBC-AA increased, while infant RBC-AA decreased. Maternal and infant RBC-DHA was higher in the sequence Sengerema (high fish)>Pare (intermediate fish)>Maasai (low fish). In contrast to RBC-AA, maternal and infant RBC-DHA were intimately related. Biomagnification of DHA occurred up to a maternal RBC-DHA of 5.6 g% at delivery; from this turning point DHA became 'bioattenuated'. From delivery to 3 months postpartum, maternal RBC-DHA decreased, while infant RBC-DHA decreased in Maasai (low fish), remained constant in Pare (intermediate fish) and increased in Sengerema (high fish). Postpartum infant RBC-DHA equilibrium was reached at an infant RBC-DHA of about 5.9 g% at delivery, corresponding to a maternal RBC-DHA of 6.1 g% during pregnancy and at delivery.

Arachidonic acid (AA)

The higher RBC-AA contents in Maasai, compared to Pare and Sengerema mothers (Figure 1A), are

likely to be caused by their higher intakes of AA from meat. Biomagnification of AA[12] is consistent with data from many others studying plasma PL[14,30] or RBC.[31-33] Higher infant compared to maternal RBC-AA at delivery was previously found by us in Dominica[34] and by others in the Netherlands[32] and the data of those 2 studies fitted well within the present study (Dominica: mean maternal RBC-AA 12.4 g%, infant 16.6 g%; Netherlands: mean maternal RBC-AA 10.0 g%, infant 14.2 g% AA). The uniformly high infant RBC-AA at delivery occurred despite between-tribe differences in maternal RBC-AA and DHA (Figure 1 A and B), and in the absence of a relation between maternal and infant RBC-AA (Figure 2A).

Although we observed no consistent changes in maternal RBC-AA during pregnancy, maternal RBC-AA was consistently higher after 3 months of exclusive lactation as compared to delivery, which coincided with a drop of infant RBC-AA. The decreasing maternal AA status during pregnancy found by Al et al.[14] and the well known postnatal drop of infant RBC-AA,[35] suggest that the fetus accretes AA at the expense of maternal AA status. However, no clear relation between maternal and infant RBC-AA could be demonstrated in our data, which questions the causality of these opposing changes. The increasing maternal AA status after delivery may derive from the discontinued utilization of AA by the placenta or discontinued AA transport to the fetus.[36,37] Secondly, the postpartum increasing maternal AA status may result from decreasing maternal adipose tissue lipolysis and decreasing *de novo* lipogenesis (DNL), secondary to the changing hormonal milieu after delivery, notably the restoration of the state of diminished insulin sensitivity/compensatory hyperinsulinemia in the end of pregnancy.[38,39] Discontinuation of the influx of both of these sources of SAFA, MUFA and PUFA (i.e. notably linoleic acid) leads to less dilution of LCP.[38,39] The decrease of infant RBC-AA may have been caused by postpartum changing infant RBC-PL species[40] and the interrupted transplacental AA transport.[38,39] It can additionally be explained by a lower conversion of LA to AA, since the infant's capacity to synthesize LC-PUFA decreases drastically after delivery.[41] This lower conversion might result from lower activities of the delta-5 and delta-6 desaturase enzymes, secondary to the changing hormonal milieu after delivery. Finally, LA intakes correlate inversely with RBC-AA.[42] Consequently, the high LA content of human milk (*Kuipers, unpublished*) and the ensuing postnatal surge in the infant LA status[37] suggest that the infant's RBC-AA decrease may also be a result of the high LA intake from breast milk.

At 3 months of age, infant RBC-AA in Pare and Sengerema was comparable to that of their mothers, indicating a rapid postnatal adaptation of RBC-AA to adult levels. Taken together, AA biomagnification seems to aim at a uniform, high infant AA status during pregnancy, most likely to sustain neurodevelopment and infant growth.[43] A high and uniform AA status seems to be subject of declining importance after delivery, which is supported by the maternal data.

Docosahexaenoic acid (DHA)

As expected,[44] maternal DHA status, and thereby infant RBC-DHA status, appeared highly sensitive to maternal fish intake. The virtually constant maternal RBC-DHA levels during pregnancy are in

accordance with earlier data for plasma PL.[14,45]

At delivery, infant RBC-DHA appeared higher than maternal RBC-DHA, but this 'biomagnification' occurred only up to about 5.6 g% maternal RBC-DHA. Beyond this point of maternal-fetal equilibrium, infant RBC-DHA was mostly lower than maternal RBC-DHA, suggesting 'bioattenuation', rather than biomagnification (Figure 2B). The mean RBC-DHA data for a Dutch population[32] (with low fish intakes) and a Dominican population[34] (Caribbean Sea; with local high fish intakes) are consistent with the switch from biomagnification to bioattenuation at a higher maternal RBC-DHA status (Dominica: mean maternal RBC-DHA 7.6 g%%, infants 6.5 g%%; Netherlands: mean maternal 3.9 g%, infants 4.7 g%). In other words, biomagnification might be confined to populations with low maternal DHA status, such as typically encountered in most Western countries, but also the currently studied Pare (intermediate fish) and Maasai (no fish), who, in contrast to the high fish consuming Sengerema women, exhibited tendencies of increasing RBC-DHA during pregnancy (Figure 1 B). Increasing amounts of DHA in the maternal circulation during pregnancy[46,47] might derive from an insulin-induced augmented elongation/desaturation of ALA in the maternal liver, secondary to the state of reduced insulin sensitivity/compensatory hyperinsulinism that is characteristic for the third trimester.[38,39] This maternal DHA increase is unlikely to occur at high fish intakes such as in the women from Sengerema, because of the negative feedback of dietary DHA on delta-5 and delta-6 desaturase activity (Figure 1B). The DHA increase in the maternal circulation might be a driving force in biomagnification that is further supported by selective transport to the fetal circulation.[48] Fetal albumin has been regarded as a major contributor to LCP biomagnification because of its ability to bind placentally transferred free LC-PUFA, while fetal albumin concentrations at term are also 10-20% higher, and albumin's free fatty acid saturation is four times lower, in the fetal circulation compared to the maternal circulation.[49] A role of the uniquely present alpha fetoprotein (AFP) with similar free fatty acid loading capacity in the fetal circulation was dismissed because of its 1,000 times lower concentration compared to albumin in the fetus.[49] AFP, however, has high preference for AA and DHA and is preferentially to albumin taken up by immature tissues,[50,51] suggesting that, rather than concentrations, notably fluxes of LC-PUFA trapping proteins might be important for biomagnification. Another suggested mechanism for the trapping of LC-PUFA is the incorporation of free LC-PUFA from the fetal circulation into phospholipids in the liver or other organs.[49]

Higher maternal compared to infant RBC-DHA contents were previously noted after daily supplementation of pregnant women from 20 weeks of gestation until delivery with a high fish oil supplement containing 2.24 g DHA and 1.12 g EPA.[52] In this study a maternal-fetal equilibrium was found at an RBC-DHA of 8.87 g%, named 'saturation point'. A possible explanation for the equilibrium at higher maternal RBC-DHA than the current about 6 g%, is that fish oil was supplemented during the 3rd trimester of pregnancy, which is characterized by adipose tissue mobilization.[53] Therefore, the supplemental DHA might have been abundantly available for incorporation into circulating lipids, and to a lesser extent adipose tissue stores, which might have resulted in an overestimation of the genuine DHA status during this non-steady state condition characterized by preferential

nutrient transfer to the rapidly growing infant. In our study, circulating DHA derived from lifelong stable fish intakes that probably caused a steady-state DHA supply of the infant from both the diet and adipose tissue stores.

Data from our populations with stable high intakes of fish suggest that DHA bioattenuation aims at the inhibition of abundant transplacental passage, possibly to prevent undesired competition of DHA with AA in competition-sensitive fetal organs. Consistent with this notion, we have shown that at high DHA status, DHA seems to lower AA, at least in postnatal RBC, and that in umbilical vessels DHA does not seem to exceed certain levels, possibly to avoid competition with AA.[54] Prevention of DHA competition with AA in the fetal period seems important, since fetal AA is implicated in growth.[10,43,55]

The consistent decrease of maternal RBC-DHA during lactation suggests that a lactating woman loses large quantities of DHA to her infant via the milk. This postpartum mother-to-infant DHA 'surge'[37] may represent a genuine form of 'postnatal DHA biomagnification', since it clearly occurs at the expense of the mother. It coincides with a more rapid postnatal accretion of DHA in the infant brain compared with AA,[56] and may therefore illustrate the increasing importance of DHA for retinal and neurodevelopment[56,57] after delivery.

The mother-to-infant DHA surge via the milk seemed unable to prevent a RBC-DHA drop in the Maasai, prevented a decrease in the Pare infants, but did enable a postpartum RBC-DHA increase in the infants of the Sengerema women (Figure 1B). A state of postpartum infant DHA equilibrium can therefore be reached at lifelong DHA intakes that are somewhat below the intakes by the Sengerema women, who reported to eat fish 4-5 times/wk on average. Using the joined data of all mothers and infants, we estimated that mothers with an RBC-DHA status of about 6 g% in early pregnancy will have an RBC-DHA of about 6 g% at delivery and will give birth to infants with about 6 g% RBC-DHA. Whether this maternal RBC-DHA of 6 g% constitutes an appropriate target for the DHA status of the mother is questionable, since a maternal RBC-DHA of 6 g% is unable to prevent a drop in maternal RBC-DHA during lactation. We[37] recently showed that maternal postpartum DHA-equilibrium is reached at a maternal RBC-DHA content of 8 g% DHA, which is even higher than the mean DHA status of the pregnant women in Sengerema, and coincides with an increase of infant RBC-DHA from 7 g% at delivery to adult levels of 8 g% at 3 months.

We recently estimated that our hunter-gatherer ancestors living in a water-land ecosystem had daily intakes of gram amounts of AA, EPA and DHA,[7] which are substantially higher than the current daily intakes of about 200 mg AA and 275 mg DHA (men) from a typically Western diet.[8,58] There is also good evidence showing that daily intakes above the recommended 450 mg DHA+EPA might be beneficial for the lowering of heart rate, blood pressure and triglycerides, and to reach maximum antithrombotic effects.[59] It has been proposed that optimal protection from cardiovascular disease occurs from 8 g% RBC EPA+DHA,[60,61] while an RBC-DHA of 7 g% [i.e. RBC-(EPA+DHA) ≥8 g%], as found in healthy subjects in Japan, might be an appropriate target to minimize major depressive disorders and bipolar depression.[62] The above mentioned maternal postpartum equilibrium of 8

g%[37] is in line with these suggested targets for adequate DHA status and also the observation that antagonism between EPA+DHA and AA occurs at RBC-(EPA+DHA) contents above 8 g%.[54]

Pregnant women in Western countries, with low intakes of fish have RBC-DHA contents well below 8 g%. For example, non-supplemented Dutch pregnant women had RBC-DHA contents of 4.41 g% at 36 weeks,[19] while after 12 weeks of exclusive lactation their infants had an RBC-DHA content of 4.73 g%. Supplementation of 220 mg DHA or 220 mg DHA + 220 mg AA during pregnancy resulted in a maternal RBC-DHA contents of 5.51g% and 5.57 g% at 36 weeks gestation, respectively,$_{19}$ while their exclusively breastfed infants had RBC-DHA of 5.50 g% and 4.95 g%, respectively, after 12 weeks. The mean daily intake of EPA+DHA by Dutch women amounted to 84 mg in 2003.[63] In another study, daily supplementation of 1.3 g DHA during 3 months of lactation resulted in a maternal RBC-DHA of 7.9 g%[64] that coincided with an infant RBC-DHA content of 9.1 g%[65] Taken together, these data suggest that the presently advised intake of 200-300 mg DHA daily during pregnancy and lactation[66] will be insufficient to reach the maternal RBC-DHA target of 6 g% (i.e. postpartum infant equilibrium) and will be grossly insufficient to reach the target of 8 g% RBC-DHA (i.e. postpartum maternal equilibrium and infant increase).

Limitations

It should be noted that, because of local constraints, the data for each tribe were derived from different subgroups that were studied at various occasions during pregnancy and lactation. We interpreted differences between these subgroups in terms of longitudinal changes, as based on between-group statistical differences. This reasoning seems justified because of their stable dietary intakes. In addition, the courses of RBC-AA and DHA during pregnancy and lactation were comparable with those of Dutch women, as determined in plasma-PL.[14] Another limitation might be the use of RBC-LCP data as proxies for whole body LCP status. Their use is supported by animal experiments[67] and therefore RBC-AA and DHA contents are nowadays widely regarded as reliable parameters for LCP-status.[68] There are, however, few data at very high DHA intakes on the efficiency of DHA incorporation in the various compartments (e.g. brain), and it may be expected that each of these compartments will ultimately become 'saturated' to reach compartment-specific DHA saturation levels, as e.g. suggested by the DHA contents of umbilical arteries and veins.[54] The difficulty to include women in their first trimester of pregnancy resulted into a small sample size. Few women attend to local hospitals in early pregnancy. Because of the low numbers we refrained from statistical analyses of these data. Also, the DHA status during pregnancy was previously shown to be influenced by the number of previous pregnancies.[29] However, in the present study we found no significant DHA status differences in women with 1, 2 or multiple previous pregnancies (data not shown). Finally, the LC-PUFA status of the various tribes might be influenced by genetic variants (e.g. FADS 1 and FADS2 polymorphism), but these were not taken into account in the present study.

CONCLUSIONS

From the present data from populations with lifelong stable dietary habits, we conclude that infant AA status at delivery is uniform, high, and unrelated to maternal AA status, which might indicate the importance of fetal AA for growth and brain development. The DHA status of mother and infant on the other hand, are intimately related and strongly dependent on the DHA intake from fish. Beyond a maternal steady-state RBC-DHA of about 6 g%, biomagnification turns into bioattenuation, which may aim at the prevention of DHA competition with AA in infant organs that are sensitive to DHA vs. AA competition. A maternal RBC-DHA of 6 g% from early pregnancy up to delivery predicts an infant RBC-DHA of about 6 g% at delivery, which will not change during subsequent exclusive breastfeeding. Infant equilibrium, however, coincides with maternal DHA losses during lactation. The mother-to-infant DHA surge during lactation may represent the genuine form of biomagnification. Bioattenuation, rather than biomagnification, might be the physiological standard for humans, because of our ancestry from the land-water ecosystem and since studies on the prevention of cardiovascular disease and depression indicate optimal RBC-DHA contents above 7 g%. It is important to stress that all of the present data derive from populations with stable dietary habits and may not be applicable to the state of non-equilibrium that is typical for the so far conducted short term supplementation studies with DHA and AA during pregnancy and lactation.

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CHAPTER 4.2

Maternal DHA equilibrium during pregnancy and lactation is reached at an erythrocyte DHA content of 8 g per 100 g fatty acids

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ABSTRACT

Introduction. Low long-chain polyunsaturated fatty acid (LC-PUFA, LCP) consumption relates to suboptimal neurodevelopment, coronary artery disease and (postpartum) depression. Maternal-to-infant LCP-transport during pregnancy and lactation is at expense of the maternal status, a process known as biomagnification. Despite biomagnification, maternal ánd infant LCP-status generally declines during lactation.

Materials and Methods. To assess the 1) turning-point of biomagnification (level from which maternal (m)docosahexaenoic acid (DHA)-status exceeds infant (i)DHA-status), 2) 'DHA-equilibrium' (steady-state-level from which mRBC-DHA stop declining during lactation), 3) corresponding iDHA-status, 4) relationship between RBC-DHA and RBC-arachidonic acid (AA), we measured RBC-FA in 193 Tanzanian mother-infant pairs with no, intermediate (2-3 times/wk) and high (4-5 times/wk) fresh-water fish-consumption, at delivery and after 3 mo of exclusive breastfeeding.

Results. At 3 mo, mRBC-DHA was lower than the corresponding iRBC-DHA upto a mRBC-DHA of 7.9 g%. mRBC-DHA-equilibrium, with equivalent mRBC-DHA at both delivery and at 3 mo postpartum, occurred at 8.1 g%. This mRBC-DHA-equilibrium of 8.1 g% corresponded with an iRBC-DHA of 7.1-7.2 g% at delivery that increased to 8.0 g% at 3 mo. We found between-group differences in mRBC-AA, however, no differences in iRBC-AA were observed at delivery or 3 mo. Relations between RBC-DHA vs. RBC-AA were bell-shaped. **Discussion.** At steady-state LCP intakes during lactation, 1) biomagnification occurs upto 8 g% mRBC-DHA, 2) mRBC-DHA-equilibrium is reached at 8 g%, 3) mRBC-DHA-equilibrium corresponds with iRBC-DHA of 7 g% at delivery and 8 g% after 3mo, 4) unlike RBC-DHA, mRBC-AA and iRBC-AA are independently regulated in these populations, 5) bell-shaped RBC-DHA vs. RBC-AA-relations might support uniform iRBC-AA. We conclude that 8g% mRBC-DHA might be optimal for infant neurodevelopment and adult cardiovascular-disease incidence.

INTRODUCTION

The long-chain polyunsaturated fatty acids (LC-PUFA, short: LCP) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) are structural components of membrane phospholipids (PL), modulators of gene expression and precursors of eicosanoids (AA, EPA), resolvins (AA, EPA, DHA) and (neuro)protectins (DHA).[1] DHA and AA are notably abundant in the central nervous system and play important roles in fetal and infant neurodevelopment.[69] Horrobin et al.[70] suggested that collaboration between EPA and AA plays a key role in the beneficial effects of low-dose EPA in psychiatric disease. Low DHA in the central nervous system has been suggested to decrease DHA turnover, with reciprocal increase in AA turnover.[71] High-DHA intakes reduce AA in e.g. RBC[72] and possibly in brain.[73] Low (n-3) LCP status is intimately related to cardiovascular and psychiatric diseases at adult age.[74] Most populations living in Western countries are characterized by low intakes of (n-3) LCP (especially EPA and DHA), which contrasts with the derivation of our ancestors from a land-water ecosystem with abundantly available (n-3) LCP and (n-6) LCP (especially AA).[7,75]

With advancing gestation, the pregnant mother increasingly transfers LCP, notably DHA, to the developing infant. Compared to its mother, infant plasma lipids and erythrocytes (RBC) in the second half of pregnancy contain higher relative amounts of LCP, which has been coined 'biomagnification'.[13] We have recently shown that biomagnification of DHA might actually reflect low maternal (n-3) LCP status, since infants born in a population with lifetime high freshwater fish intakes have lower cord blood RBC-DHA compared with their mothers. We found that this 'bioattenuation' occurs from a maternal RBC-DHA at term of about 6 g%, which corresponds with a maternal RBC-DHA of about 6 g% in early pregnancy.[36] After birth, breastfed infants in Western populations show consistent decreases of RBC-DHA.[76,77] This might, analogous to the intrauterine period, reflect low maternal (n-3) LCP status, since infants born to mothers with lifetime high intakes of freshwater fish exhibit postpartum RBC-DHA increases.[36]

Although the transfer of nutrients via lactation is generally considered to be beneficial for the infant, it may diminish maternal stores, resulting in the so called 'maternal depletion syndrome'.[78] Maternal LCP depletion, which affects DHA more than AA, may be most pronounced with longer gestation, short birth intervals, increasing parity and multiple pregnancy.[29,79] While AA in maternal plasma PL and RBC-AA increase to prepregnancy values after delivery, there is a consistent postpartum decrease of maternal plasma PL- and RBC-DHA in lactating compared to non-lactating women.[15] Low intakes of (n-3) LCP during pregnancy were reported to result in slightly shorter gestation, marginally lower birth weight and increased risk of preterm delivery.[80] Low maternal DHA status in populations with low seafood consumption, has also been associated with a higher incidence of postpartum depression.[81] The causality is however uncertain, since randomized controlled trials (RCT) with DHA in pregnancy and lactation have been inconclusive.[82,83] For instance, in a placebo controlled trial, supplementation with 200 mg DHA/d for the first 4 mo after delivery prevented postpartum decline in plasma PL-DHA, but did not influence postpartum depressive symptoms.[82]

A recent small trial showed beneficial effects of (n-3) LCP supplementation on depression during pregnancy.[84] Finally, a declining maternal DHA status during pregnancy was suggested to be involved in compromised maternal selective attention, a key component of cognition.[85]

We investigated the transplacental LCP-gradient and the courses of maternal and infant RBC-LCP during lactation in three rural African tribes with constant, lifetime low, intermediate and high intakes of tropical freshwater fish. We were particularly interested to determine 1) the point where biomagnification turns into bioattenuation, i.e. the level from which the maternal LCP-status exceeds the infant LCP-status 2) the DHA status at which the mother reaches a postpartum RBC-DHA equilibrium, i.e. the steady-state maternal RBC-DHA level at delivery that suffices to prevent a decline in the maternal DHA status during subsequent lactation, 3) the postpartum course of infant RBC-DHA that corresponds with maternal postpartum DHA equilibrium and 4) the relation between RBC-DHA and RBC-AA at low and high fish intakes.

PARTICIPANTS AND METHODS

Study design

We studied the transplacental LCP gradient at delivery and the course of the maternal and infant LCP status during exclusive lactation. For this we selected 3 ethnic groups in Tanzania with different intakes of (n-3) LCP from local fish, i.e. the Maasai (no or low fish intake), participants from the Pare Mountains (intermediate fish intake) and participants from Sengerema (high fish intake). Each of these groups was considered homogeneous with respect to ethnicity/tribe and their lifetime dietary habits. The data for the transplacental gradient were derived from 3 groups of healthy and well-nourished mothers who delivered apparently healthy infants at term (37-42 wk). Data on the course of the LCP status in mothers and infants during exclusive lactation were derived from the comparisons of the LCP status of these mothers/infants with those of counterparts at 3 mo postpartum (PP). The latter were healthy, well nourished and had delivered an apparently healthy mature (37-42 wk of gestation) infant 10-18 wk prior to their visit to the local hospital or dispensary for the follow-up of their infant in the pediatric department. All women gave their informed consent. The study was approved by the National Institute for Medical Research in Dar-es-Salaam (NIMR/HQ/R.8a/Vol. IX/800, dated April 8, 2009) and was in agreement with the Helsinki declaration of 1975 as revised in 2000.

Study groups

The Maasai are a group of Nilotic pastoralists from the Maasai Steppe nearby the dispensary in Ruvu. Their diet is composed of curdled milk, some ugali (corn porridge) and meat. Because fish are considered as snake-like creatures, they are usually not eaten. The Pare group included Bantu women from the agricultural Pare and Sambaa tribes who visited Same hospital from the nearby Pare Mountains. Their staple foods are ugali, rice and cornwheat pancakes, with some meat, fish and beans, together with a relatively abundant consumption of fruits and vegetables. Fish consumption

is irregular since nearest lake is located >30 km from their villages. The Sengerema group included Bantu women from the fishery families living at southern shore of Lake Victoria who attended to the hospital of Sengerema. Ugali, cassava root and plantain are staple foods, but consumption of fish is markedly more regular in the Lake Victoria area than for inhabitants of the Pare area. Smoking and alcohol consumption is very uncommon within these communities, especially among women. Importantly, included participants had neither the possibility to (Pare and Sengerema), nor major interest in (Maasai), deviation from their cultural habits, including their diets. We observed, and local doctors, nurses and participating women confirmed, that neither pregnancy nor lactation is associated with any change in dietary habits in any of the investigated groups. The average dietary intakes of mothers at delivery as well as at 3 mo PP were therefore likely to be representative for the lifetime dietary habits of their respective ethnic group/tribe as a whole. Data on age, parity, fish consumption and duration of lactation were obtained from the medical records or by interviews in Kiswahili. Gestational ages were checked by the last known menstrual period and fundal height. After delivery all infants were routinely checked for signs of prematurity.

Samples and analyses

About 4 mL EDTA-anticoagulated venous blood samples (7.2 mg of sprayed K_2EDTA; in 4 mL tubes; BD Vacutainer, Plymouth, UK) were collected from the mothers at delivery and after 3 mo; and from the umbilical vein at delivery. An about 250 µL EDTA-anticoagulated blood sample (250 µL pediatric MiniCollect K_3EDTA-tubes; Greiner Bio-one, Kremsmünster, Austria) was taken from the 3 mo old infants via a heel prick. Samples were stored at 4°C in the dark and processed within 2 h after collection. RBC were separated from plasma by centrifugation and washed three times with 0.9% NaCl. After washing aliquots of 200 µL of RBC suspension (4 ml tubes) or the entire RBC-suspension (250 µL tubes) were pipetted into a teflon-sealable Sovirel tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and 50 µg 17:0. In this ready-to-transmethylate mixture FA are stable at room temperature and in the dark for months. All samples were transported at room temperature to the University Medical Center Groningen (the Netherlands) for FA analysis. FA were analysed according to previously described methods.[28] In short, analyses of FA methyl esters (FAME) were performed by capillary gas chromatography/flame ionization detection following the addition of an internal quantification standard (17:0), transmethylation and extraction. FA were quantified on the basis of the added 17:0. Fatty acid compositions are expressed as g/100g FA (g%) for consistency with current literature and the omega-3 index.

Statistics

Statistical analyses were performed with SPSS version 16.0.1 (SPSS Inc, Chicago, IL). Between-group analyses were tested for normality and subsequently analyzed with an ANOVA and student t-test or a Kruskal-Wallis test and a Mann Whitney U-test. Differences were considered significant at $P<0.05$. Differences between mothers and infants were tested with a paired-samples t-tests or a Wilcoxon

test. Differences were considered significant at $P<0.05$. Bonferroni corrections were made for type-1 errors by dividing all *P*-values by the number of comparisons. Consequently, all differences with $P<0.05$ divided by the number of comparisons, were considered significant. Correlations were tested by linear regression analysis.

RESULTS

Anthropometrics and fish intakes

We included 193 women/infant pairs in this study: 83 at delivery (63 complete pairs: 6 Maasai, 24 Pare; 33 Sengerema) and 110 after 3 mo of exclusive lactation (104 complete pairs: 8 Maasai, 36 Pare; 60 Sengerema). Drop out was due to obtaining consent for only the mother or the infant, early discharge, and logistical and analytical imperfections. The anthropometrics and characteristics of the investigated mothers and their children are shown in **Table 1**.

There were no between-group differences for the mothers at delivery or at 3 mo PP, except for a lower weight at delivery ($P<0.05$) and higher length at all times ($P<0.05$) of Maasai mothers, compared to Pare mothers, and a higher BMI of Sengerema mothers compared to Maasai at 3 mo PP ($P=0.048$). The principal relevant between-group maternal difference was in fish intakes: fish intakes of the Sengerema mothers at delivery and at 3 mo PP (4-5 times/wk) were higher than the Pare mothers (2-3 times/wk, $P<0.03$), which in turn were greater than in the Maasai mothers (0 times/wk, $P<0.004$).

Table 1. Anthropometrics of mothers and infants at delivery and at 3 months PP[1]

	Maasai	Pare	Sengerema
Delivery			
Maternal age, *y*	23 ± 4 [8]	25 ± 7 [31]	24 ± 7 [34]
Postpartum weight, *kg*	53.0 ± 5[b] [8]	57.3 ± 8[a] [23]	54.4 ± 10[a,b] [31]
Height, *m*	1.59 ± 0.06[a] [8]	1.54 ± 0.04[b] [27]	1.56 ± 0.06[a,b] [31]
BMI, *kg/m²*	20.9 ± 2.0 [8]	23.9 ± 2.0 [21]	22.4 ± 3.3 [31]
Gravida, *number*	3 ± 1 [8]	3 ± 2 [31]	3 ± 3 [34]
Para, *number*	2 ± 1 [8]	2 ± 2 [31]	2 ± 3 [34]
Gestational age at birth, *wks*	40.0 ± 0 [1]	39.9 ± 1.5 [17]	38.9 ± 1.8 [2.8]
Fish intake, *times/wk*	0 ± 0[c] [8]	3 ± 2[b] [17]	5 ± 2[a] [30]
Infant birth weight, *g*	3050 ± 400 [8]	3115 ± 535 [26]	2947 ± 751 [34]
Gender, *% male*	38 [8]	45 [29]	58 [36]
3 months PP			
Maternal age, *y*	24 ± 4 [9]	24 ± 4 [40]	24 ± 6 [61]
Weight, *kg*	52.4 ± 3 [9]	52.2 ± 11 [40]	54.6 ± 10 [61]
Height, *m*	1.60 ± 0.03[a] [9]	1.55 ± 0.07[b] [40]	1.56 ± 0.06[a,b] [61]
BMI, *kg/m²*	20.5 ± 1.1[b] [9]	21.6 ± 4.0[a,b] [40]	22.1 ± 3.0[a] [60]
Para, *number*	3 ± 1 [9]	2 ± 1 [40]	3 ± 2 [61]
Fish intake, *times/wk*	0 ± 0.7[c] [8]	3 ± 2[b] [40]	4 ± 2[a] [61]
Infant age, *wks*	16 ± 4 [9]	14 ± 3 [40]	13 ± 2 [61]
Gender, *% male*	67 [9]	47 [40]	55 [61]

Maternal and infant RBC-AA and DHA

Maternal vs. infant RBC-DHA and AA at delivery and at 3 mo PP

At delivery, RBC-DHA in Maasai mothers was not significantly different compared to their infants, but became significantly lower (*P*=0.022) at 3 mo PP. Pare mothers tended to have lower RBC-DHA at delivery (*P*=0.091), and had lower RBC-DHA at 3 mo PP compared to their infants (*P*<0.001). Compared to their infants, Sengerema mothers had higher RBC-DHA at delivery (*P*=0.029), but lower DHA at 3 mo PP (*P*=0.001) (**Table 2; Figure 1A**).

Maasai maternal RBC-AA was lower at delivery compared to their infants (*P*=0.028), but higher at 3 mo PP (*P*=0.015). RBC-AA of Pare mothers was lower at delivery (*P*<0.001), compared to their infants, but there was no difference at 3 mo PP (*P*=0.12). RBC-AA of the Sengerema mothers was lower than that of their infants at delivery (*P*<0.001) and at 3 mo PP (*P*<0.001) (**Figure 1B**).

Delivery vs. 3 months PP RBC-DHA and AA in mothers and infants

Both Maasai mothers (*P*=0.003) and their infants (*P*=0.039) had higher RBC-DHA at delivery, compared to 3 mo PP. From delivery to 3 mo PP, Pare mothers showed a decrease in RBC-DHA (*P*<0.001), but the decrease in infant RBC-DHA during lactation was non-significant (*P*=0.162). RBC-DHA in Sengerema mothers decreased after delivery (*P*=0.001), but increased in their infants (*P*=0.027) (Figure 1A).

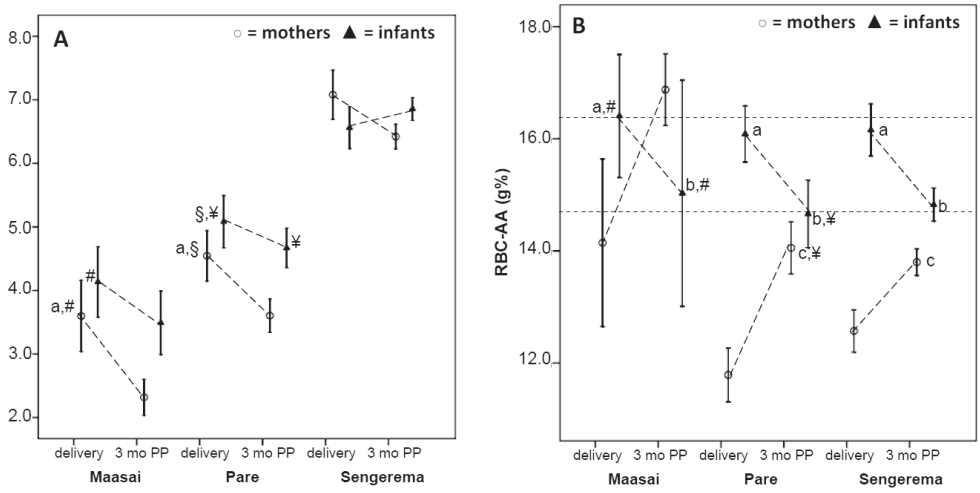

Figure 1 Maternal and infant RBC-DHA (1A) and RBC-AA (1B) at delivery and ay 3 mo PP by mothers' fish intake. Data are means ± 2*SEM. Maasai mothers (n=6 at delivery; n=7 at 3 mo PP), infants (n=8; n=6); Pare mothers (n=27; n=38), infants (n=29; n=38); Sengerema mothers (n=34; n=60), infants (n=36; n=61). Statistics: only the non-significant (P>0.05) comparisons are indicated: Maasai/Pare/Sengerema mother or infant means at a time with a common letter (a,b,c) do not differ. Mothers and infants in a group at a time with a common symbol (#,§) do not differ. Corresponding timepoints that do not differ are labelled ¥. Comparisons between mother and infant were conducted using paired analyses (n= 6 Maasai, 24 Pare, 33 Sengerema). Dotted lines indicate the minimum and maximum of the mean infant RBC-AA at delivery and 3 mo PP.

Table 2. Maternal erythrocyte fatty acids at delivery and after 3 months lactation by mothers' fish intake[1,2]

	Maasai (no fish)		Pare (medium fish)		Sengerema (high fish)	
	Delivery	3 months PP	Delivery	3 months PP	Delivery	3 months PP
	g/100 g fatty acids (g%)					
Mothers, n	6	9	27	38	34	60
SAFA	52.0 (49.5-53.7)[a]	54.4 (52.3-56.5)[d.*]	54.3 (51.0-60.2)[b]	54.8 (52.0-58.4)[d.#]	53.7 (51.6-57.5)[b]	54.4 (49.0-57.7)[d.#]
MUFA	21.4 (19.0-24.0)[a.#]	17.1 (15.5-19.5)[d.#.*]	20.3 (18.0-24.6)[a.#]	18.2 (15.7-23.6)[e.#.*]	18.2 (15.3-21.0)[b.#]	17.4 (15.3-20.6)[d.*]
PUFA	34.6 (34.1-36.5)[a]	38.7 (37.8-39.7)[d.e.#.*]	34.8 (27.6-39.3)[a]	38.1 (28.5-42.6)[d.#.*]	36.8 (33.5-40.5)[b.#]	39.4 (35.8-43.0)[e.*]
18:3(n-3)	0.23 (0.11-0.28)[a.#]	0.27 (0.20-0.34)[d.*]	0.14 (0.02-0.28)[b.#]	0.16 (0.07-0.58)[e.*]	0.14 (0.08-0.21)[b.#]	0.14 (0.09-0.71)[f.#]
20:5(n-3)	0.18 (0.13-0.29)[a.#]	0.44 (0.33-0.87)[d.*]	0.22 (0.04-0.55)[a.#]	0.39 (0.10-1.14)[d.#.*]	0.38 (0.12-1.10)[b.#]	0.64 (0.15-1.72)[e.#.*]
22:5(n-3)	1.58 (1.27-1.94)[a.b.#]	2.15 (1.46-3.46)[d.#.*]	1.38 (0.80-2.39)[a.#]	1.77 (1.18-2.80)[e.#.*]	1.64 (0.98-3.27)[b.#]	1.92 (1.47-2.93)[e.#.*]
22:6(n-3)	3.41 (2.71-4.43)[a]	2.17 (1.95-2.60)[d.#.*]	4.63 (2.76-6.83)[a]	3.52 (1.94-5.11)[e.#.*]	7.21 (4.24-9.28)[b.#]	6.46 (4.54-8.62)[f.#.*]
ΣLCP(n-3)	5.16 (4.67-6.27)[a]	5.01 (4.44-6.60)[d]	6.18 (3.60-9.51)[a]	5.54 (3.35-8.40)[d]	9.19 (5.39-13.3)[b.#]	9.00 (6.60-13.0)[e.#]
Σ(n-3)	5.41 (4.89-6.50)[a]	5.26 (4.64-6.91)[d]	6.35 (3.65-9.66)[a]	5.88 (3.54-8.57)[d]	9.34 (5.49-13.5)[b.#]	9.12 (6.70-13.2)[e.#]
18:2(n-6)	7.86 (7.19-9.94)[a.#]	10.3 (10.1-11.4)[d.*]	9.91 (6.96-13.9)[b.#]	10.9 (8.08-13.8)[d.#.*]	9.41 (6.51-13.5)[a.b.#]	10.6 (7.40-17.9)[d.*]
20:3(n-6)	2.17 (1.53-2.83)[a]	1.89 (1.47-2.19)[d.e.#]	1.63 (1.13-2.31)[a.b.#]	1.75 (1.29-2.25)[e]	1.58 (1.14-1.86)[b.#]	1.53 (1.14-2.50)[d.#]
20:4(n-6)	14.7 (11.9-15.5)[a.#]	17.1 (15.8-17.3)[d.#.*]	11.8 (8.83-13.8)[b.#]	14.4 (10.1-16.4)[e.*]	12.6 (10.3-14.9)[c.#]	13.8 (11.6-15.9)[e.#.*]
22:4(n-6)	2.87 (1.64-3.85)[a.b]	2.80 (2.12-2.99)[d]	2.75 (1.93-4.00)[a.#]	2.72 (1.62-3.64)[d]	2.38 (1.68-3.88)[b.#]	2.09 (1.39-3.70)[e.*]
22:5(n-6)	1.21 (0.73-1.82)[a]	1.00 (0.63-1.11)[d]	1.06 (0.65-1.57)[a.#]	0.84 (0.53-1.28)[d.#.*]	0.85 (0.63-1.58)[a.#]	0.76 (0.56-1.34)[d.*]
ΣLCP(n-6)	20.1 (18.1-23.4)[a.#]	22.9 (20.1-23.3)[d]	17.9 (13.9-20.5)[b.#]	19.9 (14.7-22.4)[e.#.*]	17.0 (12.9-19.3)[c.#]	18.5 (15.5-21.9)[f.#.*]
Σ(n-6)	28.6 (27.3-31.0)[a]	33.2 (30.2-34.3)[d.#.*]	27.7 (21.6-31.1)[a.#]	31.1 (24.5-35.3)[e.*]	26.3 (19.4-30.1)[b]	29.3 (24.6-34.1)[f.*]

Abbreviations: PP, pospartum; FA, fatty acids; SAFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; LC, long-chain PUFA (≥ 20 C-atoms).

[1], Values are medians (range). At a time, medians in a row without a common letter differ. [#], Different from corresponding mother/infant; *, different from delivery. All at $p<0.05$

[2], Table presents all data. These differ slightly from data for mother-infant pairs alone; significances with [#] derive from paired-analyses (n=6 Maasai, 24 Pare, 33 Sengerema)

Table 2 (continued). Infant erythrocyte fatty acids at delivery and after 3 months lactation by mothers' fish intake[1,2]

	Maasai (no fish)		Pare (medium fish)		Sengerema (high fish)	
	Delivery	3 months PP	Delivery	3 months PP	Delivery	3 months PP
	g/100 g fatty acids (g%)					
Infants, n	8	8	29	38	36	61
SAFA	52.4 (50.4-56.6)[a]	52.2 (50.5-54.7)[d]	54.5 (52.2-63.4)[b]	53.9 (51.4-67.2)[d,#]	53.0 (51.5-56.7)[a]	53.9 (51.2-59.0)[d,#,*]
MUFA	16.6 (14.8-20.7)[a,#]	19.5 (18.1-32.2)[d,#,*]	14.4 (12.5-16.5)[b,#]	17.8 (13.1-25.0)[e,#,*]	14.7 (13.0-19.6)[b,#]	17.0 (14.2-20.2)[e,*]
PUFA	34.2 (29.4-35.1)[a]	36.9 (29.4-39.3)[d,#]	34.0 (26.6-37.1)[a]	37.9 (26.3-42.4)[d,*]	35.7 (29.7-37.9)[b,#]	39.3 (34.1-46.0)[e,*]
18:3(n-3)	0.09 (0.05-0.13)[a,#]	0.23 (0.16-0.38)[d,*]	0.06 (0.01-0.14)[a,#]	0.15 (0.03-0.68)[d,e,*]	0.06 (0.01-0.13)[a,#]	0.15 (0.01-0.40)[e,#,*]
20:5(n-3)	0.11 (0.05-0.17)[a,#]	0.35 (0.15-0.49)[d,e,*]	0.12 (0.01-0.21)[a,#]	0.26 (0.03-0.57)[d,#,*]	0.19 (0.06-0.94)[b,#]	0.45 (0.19-1.30)[e,#,*]
22:5(n-3)	0.33 (0.26-0.60)[a,#]	1.27 (0.94-1.89)[d,#,*]	0.39 (0.24-0.62)[a,#]	1.27 (0.56-1.92)[d,#,*]	0.54 (0.35-2.59)[b,#]	1.23 (0.95-1.79)[d,#,*]
22:6(n-3)	4.09 (2.89-5.46)[a]	3.50 (2.08-4.12)[d,#,*]	4.99 (2.44-6.88)[b]	4.68 (2.31-6.68)[e,#]	6.44 (4.66-9.10)[c,#]	6.90 (5.10-8.62)[f,#,*]
ΣLCP(n-3)	4.48 (3.31-6.11)[a]	5.26 (3.42-5.81)[d]	5.50 (2.72-7.62)[b]	6.29 (2.90-8.89)[d]	7.12 (5.16-12.6)[c,#]	8.60 (6.24-11.2)[e,#,*]
Σ(n-3)	4.55 (3.43-6.16)[a]	5.45 (3.80-6.06)[d]	5.53 (2.79-7.69)[b]	6.51 (3.08-9.10)[e,*]	7.18 (5.17-12.8)[c,#]	8.80 (6.51-11.4)[f,#,*]
18:2(n-6)	3.01 (2.54-4.21)[a,#]	9.22 (7.23-12.4)[d,#,*]	3.21 (2.28-4.34)[a,#]	9.36 (6.47-11.8)[d,#,*]	3.37 (1.98-5.73)[a,#]	9.83 (5.78-18.4)[d,*]
20:3(n-6)	2.49 (1.81-3.29)[a]	1.65 (0.88-1.81)[d,#,*]	2.28 (1.81-3.41)[a,#]	1.77 (0.98-2.27)[d,*]	2.22 (1.60-2.75)[a,#]	1.68 (1.11-2.68)[d,#,*]
20:4(n-6)	16.6 (14.6-18.3)[a,#]	15.2 (10.4-16.4)[d]	16.2 (13.0-17.9)[a,#]	14.6 (9.10-17.4)[d,*]	16.2 (12.6-18.5)[a,#]	14.9 (10.9-16.9)[d,#,*]
22:4(n-6)	2.84 (1.69-3.70)[a]	2.44 (0.99-3.07)[d,e]	3.09 (2.34-4.01)[a,#]	2.70 (1.58-3.85)[d,*]	2.90 (1.53-4.14)[a,#]	2.01 (1.37-3.20)[e,*]
22:5(n-6)	1.82 (1.24-2.50)[a]	1.26 (0.43-1.50)[d,e,#,*]	1.67 (1.30-2.15)[a,#]	0.96 (0.50-1.64)[d,#,*]	1.37 (0.58-2.60)[b,#]	0.77 (0.48-1.30)[e,*]
ΣLCP(n-6)	24.1 (21.0-26.1)[a,#]	21.2 (13.1-23.8)[d,e,#,*]	23.7 (19.6-26.5)[a,#]	21.7 (13.4-25.0)[d,#,*]	22.0 (16.6-25.0)[b,#]	19.7 (15.0-22.6)[e,#,*]
Σ(n-6)	27.0 (23.5-29.4)[a,b]	30.9 (25.2-33.3)[d]	27.1 (22.0-30.0)[a,#]	30.7 (20.9-34.9)[d,*]	25.5 (19.6-28.0)[b]	29.8 (22.3-37.6)[d,*]

Abbreviations: PP, pospartum; FA, fatty acids; SAFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; LC, long-chain PUFA (≥ 20 C-atoms).
[1], Values are medians (range). At a time, medians in a row without a common letter differ. [#], Different from corresponding mother/infant; *, different from delivery. All at $p<0.05$
[2], Table presents all data. These differ slightly from data for mother-infant pairs alone; significances with [#] derive from paired-analyses (n=6 Maasai, 24 Pare, 33 Sengerema)

In the Maasai, maternal RBC-AA increased from delivery to 3 mo PP (P=0.003), but infant RBC-AA did not decrease (P=0.12). For the Pare, maternal RBC-AA increased (P<0.001), while infant RBC-AA decreased (P=0.001). Maternal RBC-AA increased also in Sengerema (P<0.001), and decreased in their infants (P<0.001) (Figure 1B).

Between-group differences in RBC-DHA and AA

Maasai mothers tended to have lower RBC-DHA, compared to Pare mothers at delivery (P=0.06), while Maasai infants had lower RBC-DHA compared to Pare infants (P=0.040). At 3 mo PP, both Maasai mothers (P<0.001) and their infants (P=0.004) had lower RBC-DHA compared to Pare. Sengerema mothers and their infants had higher RBC-DHA compared to Pare counterparts at delivery and at 3 mo PP (all P<0.001). RBC-DHA of Sengerema mothers and their infants were also higher compared to Maasai counterparts at all times (all P<0.001) (Figure 1A).

Maternal RBC-AA was higher in Maasai compared to Pare mothers at delivery (P=0.005) and at 3 mo PP (P<0.001), but RBC-AA was not significantly different between Maasai and Pare infants at delivery nor at 3 mo PP. RBC-AA in Sengerema mothers was higher (P=0.049) compared to Pare mothers at delivery, but there was no difference at 3 mo PP.

Also, there were no differences in infant RBC-AA between Sengerema and Pare at delivery, nor at 3 mo PP. RBC-AA was lower in Sengerema, compared to Maasai mothers at delivery (P=0.042) and at 3 mo PP (P<0.001). There were no differences between Sengerema and Maasai infant RBC-AA at delivery nor at 3 mo PP (Figure 1B).

Maternal vs. infant RBC-DHA and maternal vs. infant RBC-AA

When the 3 groups were combined, there were significant relations between maternal and infant RBC-DHA at delivery (**Figure 2A**) and at 3 mo PP (**Figure 2C**). Data that were previously reported for mother-infant pairs in Dominica[34] and The Netherlands,[32] fitted well within the relationships (Figure 2A and **Figure 2B**). Maternal RBC-DHA explained 61% of the variation in infant RBC-DHA at delivery and 76% at 3 mo PP. In contrast, there were no significant relations between maternal RBC-AA and infant RBC-AA, neither at delivery, nor at 3 mo PP (Figure 2B and **Figure 2D**). Using the equations shown on Figure 2 (at $y = x$), we calculated that at delivery, maternal RBC-DHA levels <5.6 g% corresponded with higher infant RBC-DHA, while infant RBC-DHA was lower than maternal RBC-DHA at ≥5.6 g% (Figure 2A). At 3 mo PP, maternal RBC-DHA levels <7.9 g% corresponded with higher infant RBC-DHA. There were few mothers with RBC-DHA >7.9 g%, but extrapolation suggests that the reverse may occur beyond this maternal RBC-DHA status (Figure 2C).

Maternal and infant RBC-DHA vs. RBC-AA

The relations between RBC-DHA and RBC-AA for the mothers and the infants at delivery and at 3 mo PP are presented in **Figure 3**. We first analyzed the DHA compared with AA relations in each of the 3 tribes. Linear regression analyses (**Table 3**; Figure 3) showed different slopes for the Maasai, Pare,

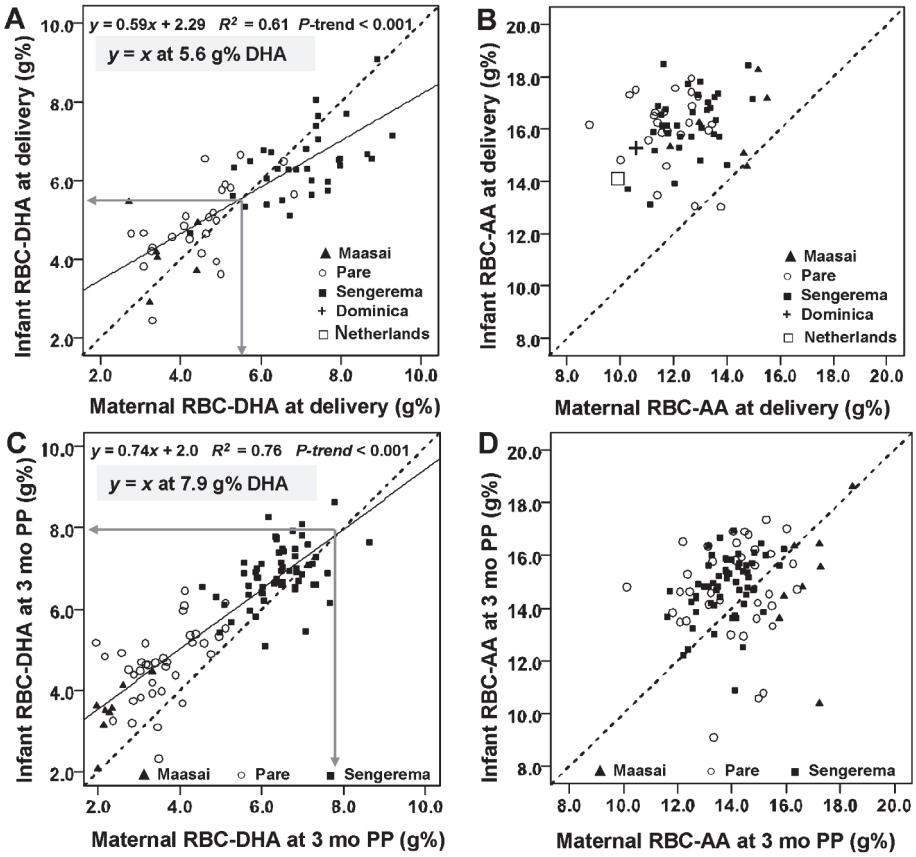

Figure 2. Relations between maternal and infant RBC-DHA (A,C) and between maternal and infant RBC-AA (B,D) at delivery (A,B) and at 3 mo PP (C,D). Mean data for Dominician (n=7) and Dutch (n=183) mother-infant pairs are derived from (34) and (32), respectively. Dotted lines represent *y* = x, i.e. the maternal RBC-DHA status equals the infants' status.

and Sengerema. Those of the Maasai and Pare were mostly positive, whereas those in Sengerema were mostly negative. Pooling of all data suggested that the relations between RBC-DHA and RBC-AA were at best bell shaped and were therefore fitted in a quadratic function (Table 3; Figure 3). The calculated curves reached their summits at 5.7, 5.7, 4.8, and 6.2 g% RBC-DHA (Fig. 3A–D, respectively). The best linear fit from a RBC-DHA above the respective summits is also in Table 3. Taken together, the relations were positive up to ;6 g% RBC-DHA and became negative or nonsignificantly positive beyond ~6 g%.

Changing postpartum maternal RBC-DHA as a function of RBC-DHA at delivery

The fractional changes of maternal RBC-DHA from delivery to 3 mo PP as a function of maternal RBC-DHA at delivery (**Figure 4A**) and infant RBC-DHA at delivery (**Figure 4B**) derive from the combined Maasai, Pare and Sengerema participants, studied at delivery and at 3 mo PP. Fractional changes are given as the ratio (maternal RBC-DHA at 3 mo PP)/(maternal RBC-DHA at delivery) in which a ratio <1

Table 3. Maternal RBC-DHA and infant RBC-DHA vs. RBC-AA at delivery and at 3 months PP[1]

Figure	Maasai linear	p[2]	Pare linear	p	Sengerema linear	p	Combined data quadratic	p	Summit of quadratic f(x)[3]	Combined data > summit (linear)[4]	p
3A	0.16x + 13.6	NS	0.47x + 9.7	0.04	-0.43x + 15.6	0.007	8.49 + 1.47x -0.13x^2	0.07	5.7	-0.61x + 16.9	0.003
3B	0.05x + 16.2	NS	0.58x + 13.1	0.007	-0.52x + 19.6	0.025	9.62 + 2.41x -0.21x^2	0.003	5.7	-0.69x + 20.9	0.008
3C	1.15x + 14.2	0.10	0.29x + 13.0	NS	-0.37x + 16.2	0.02	11.1 + 1.33x -0.14x^2	0.007	4.8	-0.31x + 15.8	0.04
3D	3.09x + 4.3	0.002	0.72x + 11.3	0.02	0.37x + 12.3	0.08	8.38 + 2.23x -0.18x^2	0.001	6.2	0.15x + 13.8	NS

[1], The correlation RBC-DHA vs. RBC-AA with the highest R^2 is shown for each separate group (linear relations); for the combined data (quadratic relations) and for all data with RBC-DHA > the summit of the quadratic function (linear relations)

[2], Significance of the relationship: models were considered significant if $p<0.05$; borderline significance is also shown ($0.10>p>0.05$); NS, $p>0.10$

[3], Calculated maximum/summit of the quadratic function, f(x)

[4], Equation of the kinear function applied to all data beyond the calculated maximum of the quadratic relation

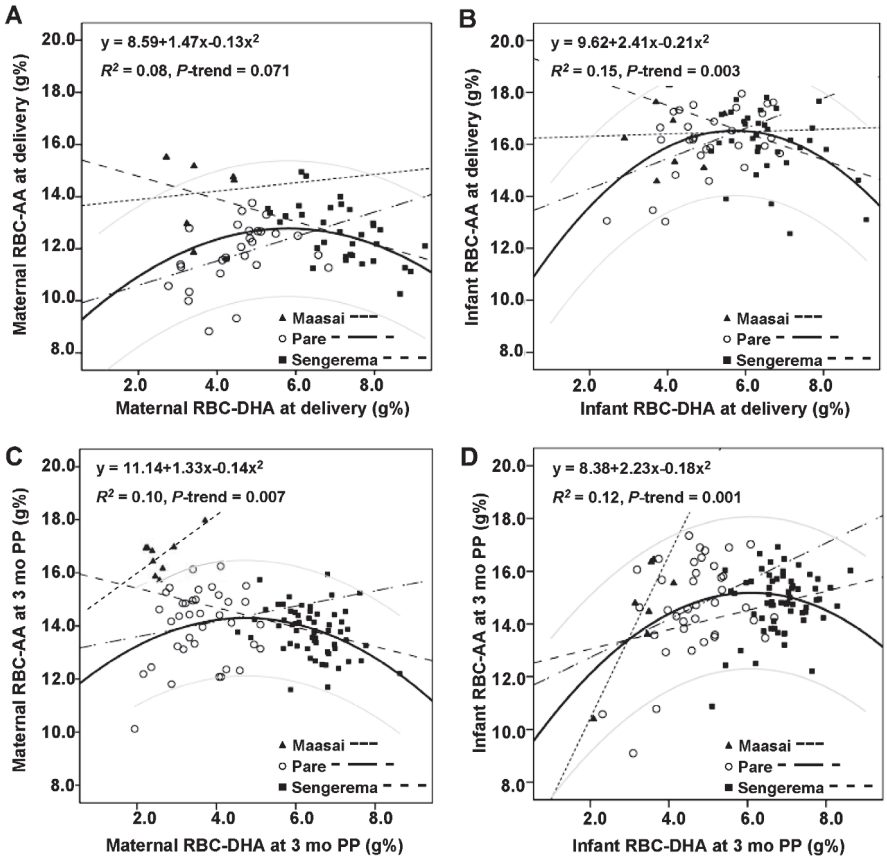

Figure 3. Relations between maternal RBC-DHA (A,C) and infant (B,D) RBC-DHA vs. RBC-AA at delivery (A,B) and at 3 mo PP (C,D). Dotted lines indicate the relation RBC-DHA vs. RBC-AA for each group. The solid lines are the quadratic functions for the combined groups with the 95% CI in gray.

indicates that the mother is in negative DHA balance during lactation and a ratio >1 indicates that the mother is in positive postnatal DHA balance. By extrapolation, it was found that a maternal RBC-DHA equilibrium from delivery to 3 mo PP was reached when the maternal RBC-DHA amounted to 8.1 g% at delivery (Figure 4A). This maternal RBC-DHA equilibrium corresponded with an infant RBC-DHA of 7.1 g% (calculated from Figure 2A) to 7.2 g% (Figure 4B) at delivery and 8.0 g% at 3 mo PP (calculated from Figure 2C). Using the 7.2 g% from Figure 4B in Figure 2A, we calculated that maternal postnatal RBC-DHA equilibrium would occur when the mother reaches an RBC-DHA of 8.3 g% at delivery, which is close to the 8.1 g% from Figure 4A.

Taken together, maternal equilibrium was reached at a maternal RBC-DHA of 8 g% at delivery, which corresponded with a maternal RBC-DHA of 8 g% at 3 mo PP, an infant RBC-DHA 7 g% at delivery that increased to an RBC-DHA of 8 g% at 3 mo PP.

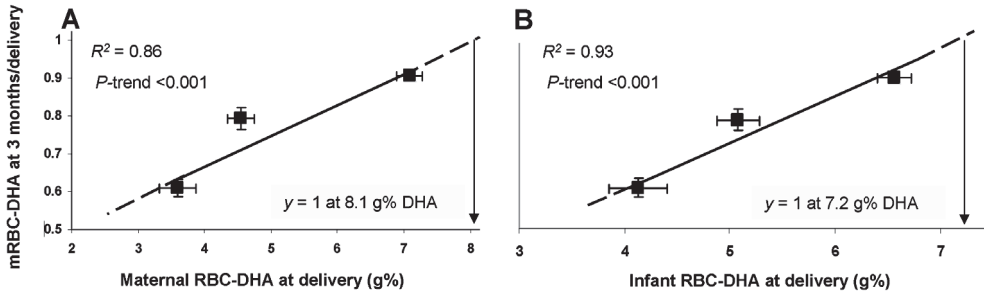

Figure 4. Maternal RBC-DHA at delivery (A) and infant RBC-DHA at delivery (B) compared with the ratio of maternal RBC-DHA at 3 mo PP/maternal RBC-DHA at delivery. Data are means ± SEM, n=6 and 9 for Maasai mothers; n=8 and 8 for Maasai infants at delivery and mo PP, respectively, n=27 and 38 for Pare mothers, 29 and 38 for Pare infants at delivery and 3 mo PP, respectively, n=34 and 60 for Sengerema mothers, 36 and 61 for Sengerema infants at delivery and 3 mo PP, respectively. A ratio of maternal RBC-DHA at 3 mo postpartum/maternal RBC-DHA at delivery <1 indicates that the mother is in negative DHA balance during lactation; a ratio >1 indicates that the mother is in positive postnatal DHA balance.

DISCUSSION

In this study of 3 populations of mother-infant pairs with different maternal consumption of freshwater fish (Fig 1A), we found that 1) biomagnification occurs up to 8 g% DHA in maternal RBC, 2) maternal RBC-DHA equilibrium is reached at 8 g%, 3) this maternal RBC- DHA equilibrium corresponds with an infant RBC-DHA of 7 g% at delivery that increases to 8 g% during 3 mo of exclusive lactation, 4) unlike RBC-DHA, maternal and infant RBC-AA are independently regulated in these populations, and 5) bell-shaped RBC-DHA vs. RBC-AA-relations might support uniform iRBC-AA.

Docosahexaenoic acid (DHA)

Although maternal RBC-DHA decreased during breastfeeding in all groups, the decline was more pronounced in Maasai and Pare, who have lower fish intakes. The postpartum change in infant RBC-DHA was dependent on the maternal RBC-DHA status and showed a decrease (Maasai), no-change (Pare) or increase (Sengerema) (Figure 1) with increasing fish intake. Using data from the same study population we recently calculated that infants would need an RBC-DHA of 6 g% at delivery to reach RBC-DHA equilibrium during lactation.[36] The present findings indicate when the mother reaches DHA equilibrium during lactation. The peculiar mother-infant sharing of DHA during pregnancy and lactation is presented in simplified form in **Figure 5**. Postnatal infant DHA equilibrium occurs at an infant RBC-DHA of about 6 g% at delivery (Figure 5A**)**, which corresponds with a maternal RBC-DHA of about 6 g% at delivery and 6 g% at early pregnancy.[36] Postnatal infant DHA equilibrium coincides with a mature milk DHA level of about 0.4 g% *(Kuipers, unpublished)*. Under these circumstances the mother loses DHA during lactation to reach The combination of infant DHA-equilibrium and maternal DHA losses during lactation can be considered as postnatal DHA-biomagnification. a maternal RBC-DHA of about 5 g% after 3 mo. This can be calculated by entering an infant RBC-DHA

of 6 g% into **Figure 3C** or Figure 4B and a maternal RBC-DHA of 6 g% into Figure 4A. Postnatal maternal DHA equilibrium is reached when maternal RBC-DHA is about 8 g% at delivery (**Figure 3A**, Figure 5b), which corresponds with 8 g% at early pregnancy[36] and a mature milk content of about 1 g% DHA *(Kuipers, unpublished)*. Under these circumstances, infant RBC-DHA is about 7 g% at delivery (**Figure 3B)**, which we named 'bioattenuation'.[36] This intrauterine bioattenuation occurs despite the rapid postnatal infant RBC-DHA increase to adult levels of 8 g% during 3 mo lactation (Figure 2C), and might illustrate the apparent importance of limiting DHA transplacental transport at high fish intakes, which is followed by a postnatal mother-to-child DHA surge via the milk.

While there is consensus on recommending an average DHA intake of at least 200 mg/d during pregnancy and lactation[80] the optimal DHA status for the mother and her infant is currently unknown. Intake of fish and fish oils during pregnancy result in slightly longer gestation, marginally higher birth weight and a reduced risk of preterm delivery.[80] However, RCTs with DHA during pregnancy targeting infant neurodevelopment are less clear. The largest RCT so far with cod liver oil (1200 mg DHA/d) on top of a baseline diet containing 200-300 mg DHA/d[86-88] showed no differences in cognitive development at 6 and 9 mo, a promising higher IQ at 4 y of age, but not at 7 y. These outcomes were in line with the negative outcomes for associations between umbilical plasma PL- and RBC-DHA and infant cognitive development at 4 and 7 y.[89,90] Supplementation with fish oil or DHA in pregnancy, however, might benefit infant visual maturation and acuity[22,91,92] and newborn sleep pattern maturity.[93] Thus, the inconsistent results of maternal DHA supplementation studies contrast with positive associations between neonatal brain DHA and cognitive and behavioral performance noted in combined human and animal studies.[69] Discrepancies might relate to e.g. short-term supplementation of relatively low doses of DHA, differences in frequencies of polymorphisms in the desaturase-enzymes and lack of dose-adjustment to differences in individual baseline maternal and infant DHA status.

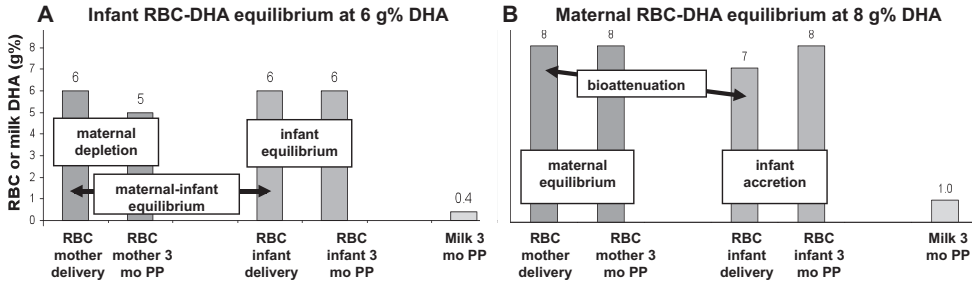

Figure 5. Synoptic overview of the infant (A) and maternal (B) RBC-DHA equilibrium during 3 mo lactation. (A) Infant RBC-DHA equilibrium occurs at 6 g% DHA and coincides with depletion of maternal DHA from 6 g% DHA at delivery to 5 g% DHA at 3 mo PP; a maternal RBC-DHA of 6 g% at 3 mo PP coincided with 0.4 g% DHA in milk. (B) Maternal RBC-DHA equilibrium occurs at 8 g% DHA, which coincides with an increase in infant RBC-DHA from 7 g% DHA at delivery to 8 g% at 3 mo PP; a maternal RBC-DHA of 8 g% coincided with 1.0 g% DHA in milk.

Epidemiological studies have linked fish consumption, EPA+DHA intakes, and EPA+DHA status, to a reduction in affective disorders, cognitive impairment, Alzheimers disease and postpartum depression.[74] An RBC-(EPA+DHA) ≥8 g%, as found in healthy Japanese, seems an appropriate target to minimize major depressive disorders and bipolar depression,[62] but the relation between (n-3) LCP and postpartum depression has not been substantiated by RCTs.[82,83] With regard to cardiovascular disease, an RBC-DHA >8 g% (omega-3 index >10 g%) was associated with the lowest risk for acute coronary syndrome and sudden cardiac death.[60,61,94] (n-3) LCP intakes >450 mg/d were shown beneficial for the lowering of heart rate, blood pressure and triglycerides and to reach maximum antithrombotic effects.[59] During 2.5 millions y of evolution our genome has become adapted to a diet high in both (n-3) and (n-6) LCP.[7,75] The presumed high LCP intakes by our ancestors, likely resulted in development of maternal stores of sufficient magnitude to prevent depletion during lactation and to sustain DHA transfer to the developing infant. The high DHA in milk and RBC that were observed in populations with high fish consumption are consistent with the high intakes of AA, EPA and DHA from our Paleolithic diets.[7,75]

It has been suggested that the RBC-membrane might reach saturation at levels between 8-10 g% DHA.[64,65] Supplementation of lactating women with doses up to 1.3 g DHA/d dose-dependently increased maternal RBC-DHA to 7.9 g%, milk DHA to 1.13 g% and infant RBC to 9.1 g% DHA. While milk DHA continued to increase, no further increase in infant RBC-DHA from about 0.8 g% DHA in milk was seen, corresponding to a maternal-infant RBC-DHA of 8-10 g%.[64,65] However, we have little data (Figure 2A and 2C) to show the relation between infant and maternal RBC-DHA beyond 8 g% DHA.

Arachidonic acid (AA)

Delivering and lactating women with low, intermediate and high fish intakes proved to have different RBC-AA status at both delivery and after 3 mo (Figure 1B), although RBC-AA increased in all maternal groups after delivery. In contrast, RBC-AA was remarkably similar for all infant groups at delivery and after 3 mo and decreased consistently after delivery (Figure 1B). Biomagnification of AA across the placenta is clearly shown in Figure 2B. However, the higher infant RBC-AA, compared to maternal RBC-AA, vanished within 3 mo of lactation (Figure 2D**)**. The concomitant increase in maternal RBC-AA might derive from discontinued utilization of AA by the placenta,[95] discontinued transport to the fetus, or both. The postpartum decrease in infant RBC-AA might be consistent with the postpartum changes in the infant's RBC-PL species.[40] Mechanistically, it might result from the discontinued AA transport across the placenta and from the change of hormonal milieu that accompanies delivery, which is likely to influence FA enzymatic activities. It was e.g. shown that the infants' LCP-synthetic activity decreases drastically after delivery.[41] Even high milk AA contents, such as in Pare (0.80 g%, *Kuipers unpublished*), were unable to prevent a decrease in infant RBC-AA. This raises the question whether milk AA is at all intended to sustain infant AA status after delivery. The remarkable between-group similarity of infant RBC-AA levels at delivery and to a lesser extent at 3 mo PP rather suggests

a well controlled infant AA status during the intrauterine period that gradually assumes adult levels after delivery. This suggestion is in line with Hsieh et al.,[96] who recently showed that central nervous system (CNS) AA levels in baboon neonates are tightly controlled at the level of incorporation or utilization, that CNS AA levels were unaffected by dietary AA and that AA decreased in all CNS structures with age.

Docosahexaenoic acid (DHA) vs. arachidonic acid (AA)

We recently suggested a synergistic relation between DHA and AA at low DHA status and an antagonistic relationship between DHA and AA at high DHA status.[54,97] Such a relationship was previously proposed by Horrobin et al.[70] Our data on the antagonistic relation between DHA and AA were a.o. based on their contents in RBC. These suggested that RBC-DHA increasingly suppresses RBC-AA from an RBC-DHA level of about 6 g%.[54,97] Interestingly, in the present study we also found synergism between DHA and AA below an RBC-DHA of about 6 g% and antagonism beyond 6 g% (Figure 3). This suggests that in Maasai and Pare infants (born to mothers with low fish intakes), biomagnification of DHA across the placenta indirectly caused a synergistic increase of RBC-AA by virtue of higher transplacental DHA transport, while in the Sengerema infants (born to mothers with high fish intakes), bioattenuation of DHA across the placenta indirectly caused a diminished antagonistic decrease of RBC-AA. In other words, both biomagnification and bioattenuation during pregnancy may aim at a certain fetal AA status. The subsequent postnatal DHA surge via the milk may be regarded as a form of postnatal biomagnification. This DHA surge was apparently unable to prevent a postnatal RBC-DHA drop in Maasai infants, caused a non-significant RBC-DHA decrease in Pare, and an increase in Sengerema infants. Analogous to the intrauterine period, the resulting low DHA status in Maasai infants might have synergistically lowered their RBC-AA, while the high DHA status of the Sengerema infants might have lowered their RBC-AA in an antagonistic manner. The presumed synergy and antagonism may in this manner have contributed to the observed low inter-individual variation of RBC-AA at 3 mo PP. It is possible that their existence illustrates the important role of AA for the developing infant during pregnancy, and the increasing postnatal importance of DHA in the suppression of AA.

It might be considered a limitation that we interpreted the differences between the subgroups at delivery and 3 mo in terms of longitudinal changes. This assumption might, however, be justified by the known and checked lifetime stable dietary intakes in these populations, while in essence no different results have been noted with literature-data deriving from genuine prospective studies. Our interpretations rest on the reliability of RBC-LCP status as a proxy for whole body LCP status. The RBC-FA composition is, however, widely considered as a reliable reflection of whole body status,[68] particularly in populations with stable lifetime dietary habits. Maternal to infant FA transport may be influenced by placental size, however we observed no between-group differences for placental size or architecture while assisting deliveries. Finally, we interpreted static values in terms of fluxes. Any of these interpretations may consequently be biased and should be confirmed by data from tracer

or other dynamic studies.

We conclude that the postnatal DHA surge via the milk represents a genuine form of postnatal biomagnification that occurs up to a maternal RBC-DHA status of 8 g%. This contrasts with biomagnification via the placenta, which occurs up to a maternal RBC-DHA status of 6 g%. This discrepancy might, together with the postnatal increase in maternal RBC-AA and concurrent decrease in infant RBC-AA, indicate a switch of the importance of AA during gestation to DHA during lactation. Unlimited DHA transfer at high maternal DHA status might be undesirable because of its antagonistic effect on AA, while the postpartum DHA surge via the milk might aim at the suppression of AA by DHA. An RBC-DHA of 8 g% in adults and the rapid postpartum increase of infant RBC-DHA to 8 g% might support infant neurological development and prevent adult diseases linked to low fish intakes. Low DHA status might be associated with diseases in the peripartum period, but this needs confirmation from RCTs targeting 8 g% RBC-DHA.

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CHAPTER 4.3

The relation between the omega-3 index and arachidonic acid is bell shaped: synergistic at low EPA+DHA status and antagonistic at high EPA+DHA status

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ABSTRACT

Introduction. The relation between docosahexaenoic (DHA) and eicosapentaenoic (EPA) vs. arachidonic acid (AA) seems characterized by both synergism and antagonism.

Materials and Methods. Investigate the relation between EPA+DHA and AA in populations with a wide range of EPA+DHA status and across the life cycle. EPA+DHA and AA were determined in erythrocytes (RBC; n=1979), umbilical arteries (UA; n=789) and umbilical veins (UV; n=785).

Results. In all compartments, notably RBC, the relation between EPA+DHA and AA appeared bell-shaped. Populations with low RBC-EPA+DHA (<2g%) exhibited positive relationships; those with high RBC-EPA+DHA (>8g%) negative relationships. Antagonism in UA and UV could not be demonstrated.

Conclusion. Both synergism and antagonism might aim at a balance between ω6 and ω3 long-chain polyunsaturated fatty acid (LCP) to maintain homeostasis. Synergism might be a feature of low LCPω3 status. AA becomes suppressed by antagonism from an RBC-EPA+DHA >8g%.

INTRODUCTION

The long-chain polyunsaturated fatty acid (LCP) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) are important for human development and health. They function as structural components of membrane phospholipids (PL), modulators of gene expression, and precursors of eicosanoids (AA, EPA), resolvins (AA, EPA, DHA) and (neuro) protectins (DHA).[1] Most Western countries are characterized by low intakes of especially EPA and DHA from fish, which contrasts with the evolution of our ancestors in a land-water ecosystem with abundantly available LCPω3 and LCPω6 (especially AA).[7,23-26] Low LCPω3 status has been suggested to be disadvantageous in infant neurodevelopment[98,99] and is associated with cardiovascular and psychiatric diseases at adult age.74 The resulting disbalance between pro-inflammatory AA-derived mediators, and the anti-inflammatory mediators from AA, EPA and DHA (1,100,101) may be an important factor in the development of a state of chronic systemic low grade inflammation, which is increasingly acknowledged to be associated with many typically Western diseases.[102] In view of the opposing roles of ω6 and ω3 fatty acids (FA) in inflammation, several authors[100,103] suggested that an increased dietary intake of ω3 FA is desirable to reduce disease susceptibility, secondary to the lowering of the AA-status.

From the majority of intervention studies that aimed at increasing linoleic acid (LA), AA and DHA intakes, it might be concluded that the relationship between ω3 and ω6 FA is mainly characterized by antagonism. However, both synergistic (positive slope) and antagonistic (negative slope) relationships between ω3 and ω6 FA have been reported in observational and ω3 FA intervention studies[70,104-111] We hypothesized that synergism between LCPω3 and LCPω6 occurs at low LCPω3 status, whereas antagonism occurs at high LCPω3 status.

Testing the suggested bell-shaped relationship between LCPω3 and LCPω6 in a cross sectional study design requires data from populations with a wide range of notably LCPω3 status. For this we composed erythrocyte (RBC) and umbilical vessel FA datasets from the various studies that we performed during the past years in many countries. The subjects were of different ages, had typically Western diets (van Goor, *unpublished*) or traditional East-African lifestyles[36,37] and were either well- or malnourished.[111,112] The dataset also contained information from supplementation studies.[19,20] In this dataset we investigated the relationship between the omega-3 index (i.e. the sum of EPA and DHA) as a marker of LCPω3 status[113,114] and the AA contents in RBC, and in umbilical veins (UV) and arteries (UA). The RBC-FA composition is a reliable reflection of dietary FA intakes[68] and umbilical vessel FA are known to be responsive to the maternal LCPω3 status.[115,116]

SUBJECTS AND METHODS

Subjects

We included subjects from Tanzania,[36,37] The Netherlands,[19,20,117-119,55] Pakistan,[111] Israel[112] and Curaçao.[115] The subjects were infants at various ages, pregnant women, mothers at delivery, mothers at 3 months postpartum, and apparently healthy males and females. The participants consumed

either a mixed diet, fish diet, or a diet that consisted of mainly milk and meat (Maasai). Some of them were supplemented with LCP.[19,20,115] The dataset consisted of 1,979 RBC samples, 785 UV and 789 UA.

The ages of the subjects, their geographical location, their study numbers and a very summarily description of the diets and supplements are shown in **Tables 1-3**.

Sample collection and analyses

EDTA-anticoagulated venous blood was collected from infants and adults. EDTA-anticoagulated cord blood was collected at delivery. The samples were stored at 4°C in the dark and processed within 2 h after collection. RBC were isolated by centrifugation and washed three times with 0.9% NaCl. After washing, 200 µl of the washed RBC suspension was transferred to a teflon-sealable Sovirel tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and 50 µg 17:0..

Umbilical cords were collected immediately after delivery. A sample of about 3 cm of the umbilical cord, located at the proximal site to the placenta, was taken and stored in saline at 4 °C until further processing. One umbilical artery and the vein were dissected from the surrounding tissue. Approximately 1 cm of tissue was collected and stored in a teflon-sealable Sovirel tube containing 2 mL of methanol-6 mol/L HCl (5:1 v/v) 1 mg butylated hydroxytoluene (antioxidant) and 50 µg 17:0. All samples were transported to University Medical Center Groningen (The Netherlands) for fatty acid analysis by capillary gas chromatography/flame ionization detection according to previously described procedures.[28] Fatty acid compositions and their ratios were expressed in g% and g/g, respectively.

Data analysis and statistics

Statistical analyses were performed with PASW version 18.0 (SPSS Inc, Chicago,IL). We subdivided the RBC EPA+DHA data in parts of 1 g% to construct a bar diagram. Group differences were studied with the aid of the Kruskal-Wallis test (non-parametric) since the FA data were not normally distributed. Between-group differences were tested with the Mann Whitney U-test at $p<0.05$. We constructed scatter plots and determined the best fitting function. The coefficient of determination (R^2) was used to estimate the extent to which a given variable was explained by another. Corrections were made for type-1 errors (i.e. Bonferroni correction).

RESULTS

Figure 1A shows the bell-shaped relationship between the omega-3 index and AA in RBC, suggesting a synergistic relationship at low omega-3 status, no relation at intermediate status and an antagonistic relation at a high omega-3 index. Excluding data of supplementation studies did not affect the bell-shape of the curve (data not shown). The synergistic part occurred at an omega-3 index below 2 g% and the antagonistic part occurred beyond 8 g%. Statistical analysis confirmed lower RBC-AA at both low and high RBC-EPA+DHA contents, since the RBC-AA of subjects with an omega-3 index of 2-8 g% was significantly different from the RBC-AA of subjects with an omega-3

Table 1: Subject characteristics, number and, erythrocyte (RBC)-LA RBC-AA and RBC-EPA+DHA content

Characteristic	Mean Age	Geographical location	RBC (n)	RBC-LA (g%)	RBC-AA (g%)	RBC-EPA+DHA(g%)	Population Diet	Suppl.	Reference
infant	0 days	Tanzania	29	3.28	16.1	5.20	mixed	none	36
infant	0 days	Tanzania	8	3.18	16.4	4.24	milk/meat	none	36
infant	0 days	Tanzania	36	3.40	16.2	6.80	fish	none	36
infant	0 days	Tanzania	27	2.45	16.1	8.64	high fish	none	in preparation
LBW infant, breastfed	10 days	The Netherlands	36	7.70	13.8	4.09	mixed	none	117
LBW infant, breastfed	20 days	The Netherlands	42	9.18	12.7	3.76	mixed	none	117
LBW infant, breastfed	42 days	The Netherlands	18	10.3	12.0	3.24	mixed	none	117
LBW infant	10 days	The Netherlands	81	6.58	13.6	4.14	mixed	none	117
LBW infant	20 days	The Netherlands	81	7.52	13.6	4.39	mixed	none	117
LBW infant	42 days	The Netherlands	81	8.77	13.8	4.46	mixed	none	117
LBW infant	1 month	The Netherlands	39	8.08	11.4	4.41	mixed	LCP 1	117
LBW intant	1 month	The Netherlands	39	8.03	12.0	4.91	mixed	LCP 2	117
infant, excl. breastfed	3 months	Tanzania	38	9.36	14.7	4.94	mixed	none	37
infant, excl. breastfed	3 months	Tanzania	8	9.00	15.0	3.79	milk/meat	none	37
infant, excl. breastfed	3 months	Tanzania	61	10.3	14.8	7.37	fish	none	37
infant, breastfed	3 months	The Netherlands	57	8.14	14.6	5.15	mixed	none	20
infants, breastfed	3 months	Israel	31	8.51	13.8	4.61	mixed	none	112
infant, excl. breastfed	5 months	Tanzania	48	6.70	14.0	9.04	high fish	none	in preparation
infant, breastfed	8 months	Pakistan	19	8.40	14.7	3.29	mixed	none	112
infant malnourished, breastfed	15 months	Pakistan	48	8.54	13.8	2.89	mixed	none	112
infant, malnourished	21 months	Pakistan	40	7.78	13.1	2.29	mixed	none	112
infant	48 months	Pakistan	6	10.8	14.3	2.82	mixed	none	112
infant, breastfed	3.5 years	The Netherlands	33	10.3	14.3	3.27	mixed	none	112

througout pregnancy	25 years	Tanzania	94	10.3	13.0	5.00	mixed	none	36
througout pregnancy	24 years	Tanzania	35	9.23	15.0	3.88	milk/meat	none	36
througout pregnancy	25 years	Tanzania	110	8.72	12.0	7.74	fish	none	36
pregnant, 16 weeks	33 years	The Netherlands	166	8.04	11.5	3.62	mixed	none	unpublished
pregnant, 36 weeks	32 years	The Netherlands	41	8.13	10.9	6.07	mixed	MUM 1	20
pregnant, 36 weeks	33 years	The Netherlands	37	8.34	11.9	5.75	mixed	MUM 2	20
pregnant, 36 weeks	33 years	The Netherlands	33	8.51	11.3	4.81	mixed	MUM 3	20
pregnant, 36 weeks	32 years	Curacao	70	10.0	13.0	4.69	mixed	none	unpublished
mother at delivery	24 years	Tanzania	27	9.96	11.8	4.77	mixed	none	36
mother at delivery	25 years	Tanzania	6	8.19	14.1	3.79	milk/meat	none	36
mother at delivery	24 years	Tanzania	34	9.30	12.6	7.50	fish	none	36
mother at delivery	26 years	Tanzania	28	6.69	10.9	9.93	high fish	none	in preparation
mother at 3 months postpartum	23 years	Tanzania	38	11.0	14.1	4.05	mixed	none	37
mother at 3 months postpartum	23 years	Tanzania	9	10.1	16.9	2.78	milk/meat	none	37
mother at 3 months postpartum	23 years	Tanzania	60	10.7	13.8	7.14	fish	none	37
mother at 5 months postpartum	28 years	Tanzania	46	6.57	12.4	8.83	high fish	none	in preparation
male and non pregnant female	36 years	Tanzania	128	10.1	14.8	3.33	milk/meat	none	unpublished
male and non pregnant female	35 years	Tanzania	15	10.7	15.8	3.42	mixed	none	unpublished
male and non pregnant female	37 years	The Netherlands	69	10.4	13.9	4.25	mixed	none	118
non-pregnant female	31 years	Tanzania	30	7.90	12.8	6.65	fish	none	unpublished

Values are mean, suppl. = supplementation, fish intakes/wk: milk/meat = 0, mixed = 1-3, fish 3-7, high fish > 7

Table 1b: Subject characteristics, number and, umbilical vein (UV) and artery (UA)-LA, -AA and -EPA+DHA content

Characteristic	Mean age (yr)	Geo-graphical location	UV (n)	UV-LA (g%)	UV-AA (g%)	UV-EPA +DHA (g%)	UA (n)	UA-LA (g%)	UA-AA (g%)	UA-EPA +DHA (g%)	Population diet	Suppl.	Ref
Delivery	26	Tanzania	27	2.36	14.7	3.04	27	1.78	12.1	3.27	vegetarian	none	unpublished
Delivery	24	Tanzania	60	3.40	14.3	3.47	60	2.25	12.8	3.93	mixed	none	unpublished
Delivery	25	Tanzania	9	2.31	14.9	3.25	9	1.56	12.1	3.46	milk/meat	none	unpublished
Delivery	24	Tanzania	118	3.13	13.9	4.66	118	2.11	11.7	5.31	fish	none	unpublished
Delivery	29	Curacao	24	2.71	16.8	5.80	24	1.79	13.1	5.99	mixed	Frisomum	115
Delivery	29	Curacao	14	2.93	16.3	5.36	14	2.08	12.8	5.44	mixed	Fish oil-1	115
Delivery	31	Curacao	19	2.89	16.4	5.47	19	1.91	13.5	5.78	mixed	Fish oil-3	115
Delivery	31	Curacao	56	2.71	16.5	4.63	56	1.72	13.3	5.02	mixed	none	unpublished
Delivery	32	Curacao	57	2.71	16.5	4.77	57	1.85	13.8	5.00	mixed	none	unpublished
Delivery	32	The Netherlands	35	2.87	16.3	4.97	35	1.97	12.2	5.05	mixed	MUM 1	20
Delivery	33	The Netherlands	37	2.61	16.6	4.87	37	1.68	12.3	4.88	mixed	MUM 2	20
Delivery	33	The Netherlands	33	2.81	16.3	4.57	33	1.97	12.7	4.61	mixed	MUM 3	20
Delivery	30	The Netherlands	296	2.59	16.6	4.37	296	1.66	12.9	4.59	mixed	none	119

Values are mean, suppl. = supplementation, fish intakes/wk: milk/meat = 0, mixed = 1-3, fish 3-7, high fish > 7

Table 3: Supplement characteristics

Supplement	fatty acid	dose/day
MUM 1	LA	535 mg
2x placebo	ALA	60 mg
	AA	0.6 mg
MUM 2	LA	274 mg
placebo +	ALA	32 mg
DHA	AA	15 mg
	EPA	36 mg
	DHA	218 mg
MUM 3	LA	46 mg
DHA+AA	ALA	7 mg
	AA	220 mg
	EPA	36 mg
	DHA	218 mg
Frisomum	LCPω3	528 mg
	EPA	293 mg
	DHA	185 mg
Fish oil-1	LCPω3	336 mg
	EPA	177 mg
	DHA	123 mg
Fish oil-3	LCPω3	1008 mg
	EPA	531 mg
	DHA	369 mg
LCP 1	GLA	0.31 mol%
	LCPω3	0.38 mol %
LCP 2	GLA	0.32 mol%
	LCPω3	0.80 mol%

index below 2 g% and subjects with an omega-3 index above 8 g% (both $p<0.001$).

In **Figure 1B** we applied the above mentioned cut-off levels. We observed a positive relationship with RBC-AA at an omega-3 index below 2 g% ($y = 7.16x + 0.39$; $R^2=0.59$, $p<0.001$), no relationship at intermediate omega-3 status ($R^2<0.001$, $p=0.78$), and a negative relationship at an omega-3 index above 8 g% ($y = -0.48x + 17.17$; $R^2=0.07$, $p<0.001$).

It was considered likely that the curve is composed of many curves, that e.g. each reflect the influence of age or altered physiology (e.g. pregnancy) and may in their own right correspond with different EPA+DHA vs. AA relationships. We therefore subsequently analysed the relation between the omega-3 index and RBC-AA in subgroups with sufficient numbers and a sufficiently wide EPA+DHA range. The bell-shaped relation between the omega-3 index and AA was also detectable in RBC deriving from Palestinian, Dutch and Tanzanian infants at 3-5 months postpartum (**Figure 2A**; $y = 8.47 + 1.92x + 0.14x^2$; $R^2=0.22$, $p<0.001$). We also found a bell-shaped relation in RBC from Dutch, Curaçaon and Tanzanian, women at delivery (**Figure 2B**; $y = 11.40 + 0.41x - 0.04x^2$; $R^2=0.11$, $p<0.001$), though at a seemingly lower AA status. Pakistani infants, who were either well- or malnourished and breastfed or formula fed, and all exhibited a very low ω3 status, showed a synergistic relation between the omega-3 index and AA in RBC (**Figure 2C**).

We finally investigated the relationships between the EPA+DHA and AA contents in umbilical veins (**Figure 3A**) and arteries (**Figure 3B**) of Dutch, Curaçaon and Tanzanian infants.

Visual inspection revealed apparent synergism at low EPA+DHA contents in UV, which was confirmed by significantly higher AA in UV with EPA+DHA contents ranging from 5.0-8.0 g% as compared to UV with EPA+DHA contents below 5.0 g% ($p<0.001$). A trend to antagonism could not be demonstrated. Also in UA, we observed a

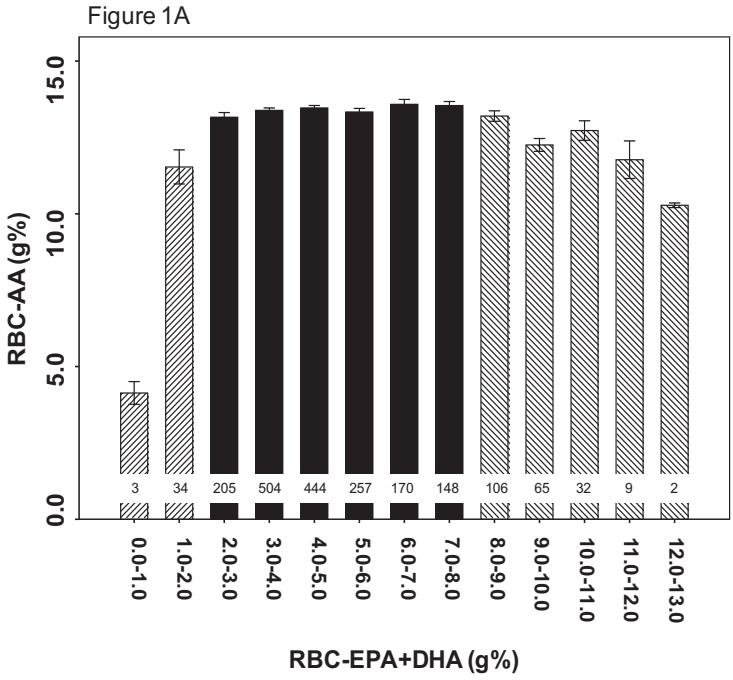

Figure 1A: Diagram representing the relationship between the omega-3 index and AA contents in erythrocytes (RBC). Different filling patterns indicate significantly different ($p<0.001$) RBC-AA contents. Fatty acid data are in g/100 g (g%). Bars show mean +/- SEM, figures indicate numbers in subgroups. Subjects were recruited in Tanzania, The Netherlands, Pakistan, Israel and Curaçao. The subjects were infants at various ages, pregnant women, mothers at delivery, mothers at 3 months postpartum, and apparently healthy males and females, supplemented and non-supplemented subjects.

bell-shaped curve, which was confirmed by significantly higher AA in UV with EPA+DHA ranging from 6.0-9.0 g% as compared to UV with EPA+DHA below 6.0 g% ($p<0.001$). The trend to a descent at higher EPA+DHA status proved statistically insignificant.

DISCUSSION

In a unique dataset from subjects with a wide range of dietary LCP intakes and status, we investigated the relationships between the omega-3 index as a marker of LCPω3 status and the AA status in RBC, UV and UA. In all instances, the relationships between the omega-3 index and AA seemed bell-shaped, suggesting that we are dealing with a general phenomenon. Synergistic and antagonistic relationships between ω3 and ω6 FA have previously been reported.[70,72,103-111,120-122] The strength of this study is the collection of samples using a single procedure, and the employment of a single gas chromatographic method for FA profiling.[28] A limitation might be the relatively poor information on the dietary compositions and intakes of the investigated subjects. However, RBC-AA and EPA+DHA are generally regarded as reliable parameters of their status[68] that are e.g. responsive

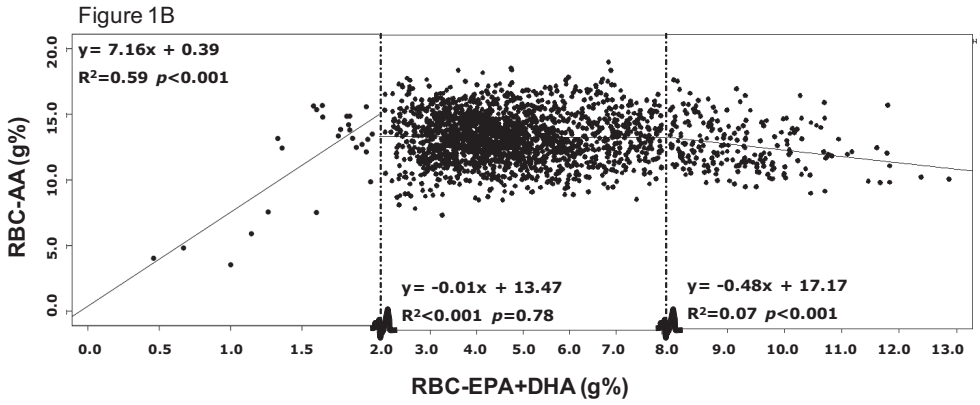

Figure 1B: Scatter plot representing the relationship between EPA+DHA and AA contents in erythrocytes (RBC). Cut-off levels are at RBC-EPA+DHA <2 g% for a synergistic relation and at RBC-EPA+DHA >8 g% for an antagonistic relationship between RBC-EPA+DHA and RBC-AA. Fatty acid data are in g/100 g (g%). Note the non-linearity of the x-axis.

to augmentation of their dietary intakes in controlled intervention studies.[72,122]

Mechanistic background of the bell-shape

In this study we concentrated on the univariate relationships between the EPA+DHA and AA status. Both the EPA+DHA and AA status might be influenced by synthesis from the parent precursors alpha-linolenic acid (ALA) and LA, dietary AA, EPA and DHA intakes and the intake of other FA by competition.[42,58,72,123-125] However, when compared with dietary EPA and DHA, supplementation of ALA hardly augments EPA and especially DHA status to a physiologically appreciable extent, even in women.[123] At high LA intakes, AA synthesis from LA increases,[58] but this remains unnoticed in RBC. On the contrary, dietary LA intake correlates inversely with AA and also with EPA and DHA, in RBC-PL.[42] Conversely, high AA intakes reduce LA in RBC.[124] The LA vs. AA relation seems therefore likely to be dominated by competition for incorporation, since at a daily intake of 15 g LA, AA synthesis falls reciprocally from 677 to 326 mg/d when AA intakes are increased from 210 mg to 1.7 g/d.[58,122] Analogously, an intake of 6.5 g DHA/d reduces the synthesis of LCPω3 from ALA and also of the LCPω6 synthesized from LA,[125] illustrating that the net effect of increased DHA or AA intakes are the reduced syntheses of both LCPω3 and LCPω6 from their respective precursors by negative feedback. The presently described bell-shaped relationship between AA and DHA may result from many factors, including their synthesis from precursors, feed-back inhibition by LCP, and competition for incorporation, both between AA and DHA and with other FA, notably LA. However, at RBC-EPA+DHA > 8 g%, RBC-AA was not correlated with RBC-LA (Pearson correlation coefficient -0.052, p=0.66), which is likely to be explained by the presence of subjects with both high and low LA status (fish Tanzania and high fish Tanzania respectively; Table 1). Thus, the complex LA vs. AA relationship is unlikely to have influenced the antagonistic part of the curve (Figures 1A and B).

The observed bell-shaped curves for RBC were likely to be the result of many bell-shaped EPA+DHA vs. AA curves, which may each be determined by specific aspects of the life cycle and notably by their corresponding EFA and LCP status. We[37] have previously shown that in different age groups, the bell-shaped relation plateaus at different AA levels. For example newborns showed a higher AA-plateau compared to their mothers and 3 months old infants. The higher RBC-AA in newborns may at least in part be caused by their lower LA status as compared with adults, which changes rapidly with the postnatal LA surge from the feeding of breast milk or infant formula.[27] We feel that we are nevertheless dealing with a general mechanism. In line with this notion we also found seemingly bell shaped relationships between DHA+EPA and AA in UV and UA. Clear antagonism as observed from an omega-3 index >8 g% in RBC was, however not observed, which is consistent with the saturation of fetal DHA with increasing maternal DHA intake and status. We reported previously that from a maternal RBC-DHA of 6 g% at birth the corresponding newborn RBC DHA is lower than that of its mother, which we named bioattenuation, as opposed to the biomagnification at a maternal RBC-DHA below 6 g%.[36] We suggested that this bioattentuation prevented competition of DHA with AA and thereby illustrates the importance of AA to the infant during pregnancy, while the postnatal DHA surge via the milk illustrates the importance of postnatal DHA status. Taken together this suggests that synergy is the standard prior to birth and that antagonism becomes important after birth.

EPA+DHA vs. AA synergism

Synergism between LCPω3 and LCPω6 was found in three ALA and LCPω3 supplemented patients with ω3 FA deficiency by Bjerve et al.[109] Payet et al.[108] showed accretion of both DHA and AA in RBC of elderly patients after consumption of DHA-enriched eggs. Synergism was also reported in a fish oil-supplemented malnourished infant[111] who, apart from an increase of RBC-DHA from 0.41 to 2.50 mol%, showed a remarkable simultaneous increase of RBC-AA from 4.04 to 13.83 mol%. Positive

Figure 2

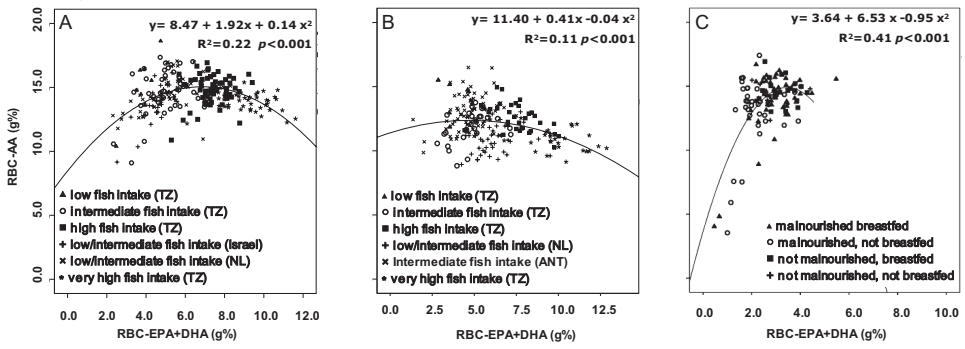

Figure 2: Scatter plot representing the relationship between EPA+DHA and AA contents in erythrocytes (RBC) of (A) 3-5 months old infants, (B) women at delivery and (C) Pakistani infants. Fatty acid data are in g/100 g (g%). TZ = Tanzania, NL = The Netherlands, ANT = Curaçao.

relations between LCPω3 and LCPω6 were observed by Desci et al.[106] in malnourished and HIV-infected infants and by Innis et al.,[110] who in non-supplemented young children reported a positive relation between DHA and 22:5ω6, which is the chain elongation/desaturation product of AA. Taken together, synergism seems to occur mainly in subjects with a discrepancy between LCPω3 need and supply.

Some of the first studies that might have shown the synergism between ω3 and ω6 FA reported that symptoms of ω6 deficiency in rats that had previously been consuming a fat-depleted diet, could be restored with 20 mg LA/d and with 10 mg LA + 10 mg ALA/d,[120] but also when only 0.5 en% LA (in stead of the usual 2.0 en% to prevent deficiency symptoms) was provided in the presence of 0.5 en% ALA.[126] Horrobin et al.[70] reported that at low intakes of EPA/fish oil, RBC membrane contents of AA rise, whereas RBC-AA decreases at high EPA/fish oil intakes. The authors[70] suggested that optimum health may require raising both ω3 and ω6 FA at low status and it is therefore likely that synergism/antagonism actually aims at a certain balance between ω3 and ω6 FA. The importance of a certain balance also emerged from the U-shaped relation between the infant RBC-DHA/AA ratio and the percentage of infants with mildly abnormal general movements.[19]

Tight regulation of the ω3/ω6 balance might have been indicated by the study of Kim et al.,[127] who showed that 15 weeks of dietary ω6 FA deprivation in rats caused downregulation of AA metabolism and upregulation of DHA metabolism in brain. Conversely, Rao et al.[71] showed that a low DHA status decreases DHA in rat frontal cortex and the expression of iPLA2, while increasing cPLA2, sPLA2 and COX-2 expression. This combination causes DHA conservation and higher AA metabolism to eicosanoids, seemingly in an attempt to preserve ω3/ω6 balance.

EPA+DHA vs. AA antagonism

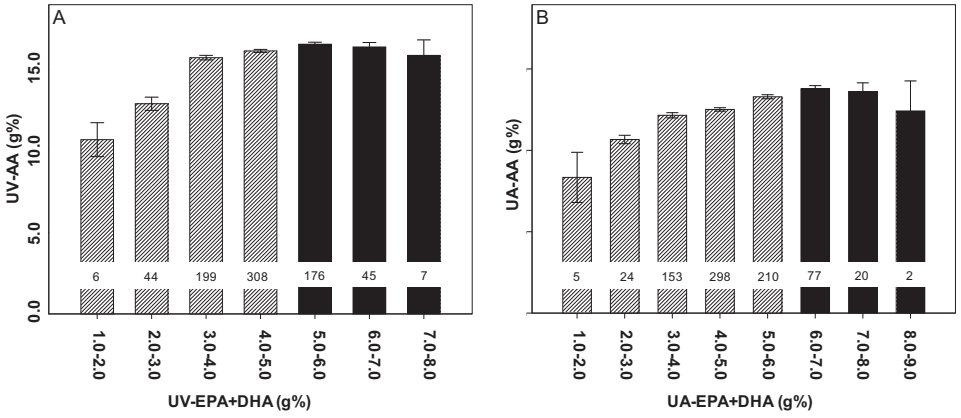

Figure 3: Bar diagram representing the relationship between EPA+DHA and AA contents of A: umbilical veins (UV) and B: umbilical arteries (UA). Different filling patterns indicate significantly different ($p<0.001$) UV/UA-AA contents. Fatty acid data are in g/100 g (g%). Bars show means +/- SEM, figures indicate numbers in subgroups. Subjects were recruited in Tanzania, The Netherlands, and Curaçao.

Many studies related a higher ω3/ω6 ratio to a decreased incidence of cardiovascular disease, autoimmune disease and psychiatric disease. The common denominator in these 'Western diseases' is a state of low grade inflammation.[6,100] The diet in most Western countries is characterized by low fish intake and a disturbed ω3/ω6 balance.[6] Higher intake of DHA and EPA is likely to result in incorporation of these FA into (inflammatory) cell membrane phospholipids, at the expense of AA. AA gives rise to eicosanoid mediators that have established roles in inflammation.[1] By both direct action and by their competition with AA, LCPω3 are known to exert an anti-inflammatory function, while LCPω6 are predominantly known for their pro-inflammatory function.[103] More recently, it was shown that the roles of LCPω3 and LCPω6 in inflammatory processes are more complex.[103] Some actions of AA-derived eicosanoids are anti-inflammatory while EPA derived eicosanoids may have the same pro-inflammatory potencies as AA derived eicosanoids. This may indicate that the complex inflammatory processes need a tight regulation that is sustained by an optimal ω3/ω6 balance.
In line with the above, the main goal of the many fish oil supplementation studies is to promote health by improving LCPω3 status, decrease AA status and eventually to improve the ω3/ω6 balance. In other words, most of them aimed at the antagonistic or competitive relationship between LCPω3 and AA. A study with patients with coronary artery disease[105] showed an increase in DHA and concurrent decrease in AA in plasma phospholipid (PL) after supplementation of 6 or 12 g ω3 FA/d for 6 months. These results were confirmed in serum lipids of healthy volunteers by DeLany et al.[107] in a 42 days supplementation study with 2 or 8 g LCPω3/d. EPA supplementation (1.35, 2.70 or 4.05 g/d for 12 weeks) of healthy young and older men by Rees et al.[104] also resulted in opposite changes in AA and EPA/DHA status in plasma phospholipids.

Optimal EPA+DHA vs. AA balance

The occurrence of a bell-shaped relation between LCPω3 and LCPω6 may reflect an attempt to adjust the AA status to the LCPω3 status. At very low LCPω3 status, AA seems suppressed to maintain the delicate balance between LCPω3 and LCPω6, whereas at very high DHA status, a second stage of AA suppression occurs. In view of many Western diseases, an omega-3 index at the antagonistic side of the curve, i.e. above 8 g% seems desirable. In healthy Japanese,[62] it was shown that an RBC-DHA of >7 g% (i.e. RBC-EPA+DHA ≥8 g%), was associated with the lowest risk of depressive disorders and bipolar depression. Protection from cardiovascular disease occurred from an omega-3 index of 8 g%.[60,61] In line with this, Kuipers et al.[37] calculated that RBC-DHA contents above 8 g% were sufficient to support a stable high maternal DHA status and an increasing infant DHA status during lactation.

There is substantial evidence that our genome evolved during millions of years of evolutionary adaptations in an East-African water-land ecosystem.[7,23-26] *Homo sapiens* might have left Africa via the shorelines[128] and lived in close vicinity of (fresh) water as a hunter-gatherer until at least 10,000 years ago.[129] The reconstruction of several possible hunter-gatherer diets in the water-land ecosystem revealed that daily intakes of DHA, EPA and AA are likely to have reached gram amounts.[7] These intakes are much higher than the recommended 450 mg DHA/d that produced beneficially

effects on cardiovascular disease in randomized controlled trials[59] and are certainly higher than the current daily intakes of about 200 mg AA and 275 mg DHA (for men) from a typically (French) Western diet.[8] The high intakes of EPA+DHA by our ancient ancestors, are in line with an outcome of the omega-3 index in the highest range,[61,94,114,130] and thereby supports a stable maternal DHA status during pregnancy and lactation and a rapidly increasing postpartum infant DHA status that reaches adult levels within 3 months.[37]

CONCLUSION

In conclusion, both synergism and antagonism between EPA+DHA and AA were observed in humans. Both may aim at a certain LCPω3/LCPω6 balance to maintain homeostasis. Synergism seems to be a feature of low LCPω3 status that in practice relates mostly to the fetus and LCPω3 deficiency states. However, from an evolutionary point of view and in line with the highest protection from psychiatric and cardiovascular disease, an antagonistic relation between EPA+DHA and AA is likely to be the physiological standard for adults. There is good evidence that the lowest risk of typically Western diseases of the heart and the brain occur from RBC- EPA+DHA contents above 8 g%.[37,60-62,114]

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CHAPTER 5

Gestational age dependent changes of the fetal brain, liver and adipose tissue fatty acid compositions in a population with high fish intake

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ABSTRACT

Introduction. There are no data on the intrauterine fatty acid (FA) compositions of brain, liver and adipose tissue of infants born to women with high fish intakes.

Subjects and Methods. We analyzed the brain (n=18), liver (n=14) and adipose tissue (n=11) FA compositions of 20 stillborn infants with different gestational ages (range 8-38 weeks) born to Tanzanian women with low linoleic acid (LA) intakes and high intakes of docosahexaenoic (DHA) and arachidonic (AA) acids from local fish.

Results and discussion. With advancing gestation, brain saturated-FA (SAFA; in g/100 g FA), polyunsaturated-FA (PUFA), DHA, 20:3ω6, 22:4ω6 and 22:5ω6 increased, while monounsaturated-FA (MUFA), 20:3ω9, 22:3ω9 and AA decreased. Decreasing brain AA might be caused by increasing AA-metabolism to 20:3ω6, 22:4ω6 and 22:5ω6. In the liver, SAFA, PUFA and LA increased, while MUFA decreased with gestation. The steep increase of (mostly *de novo* synthesized) SAFA in adipose tissue coincided with relative decreases of MUFA, PUFA, DHA, LA and AA with advancing gestation. Compared to Western infants, the currently studied African infants had higher DHA, lower AA, and a higher DHA/AA-ratio in brain and adipose tissue, while the LA content of adipose tissue was lower.

Conclusion. The low LA and high DHA and AA intakes by the mothers of these infants might support optimal α-linolenic (ALA) vs. LA competition for Δ5D and Δ6D-activities and DHA vs. AA antagonism. Conversely, the Western diet, characterized by high LA and lower DHA and AA intakes, might disturb these evolutionary conserved mechanisms aiming at an optimal ω3/ω6-balance.

INTRODUCTION

Low intakes of the long-chain polyunsaturated (LCP) fatty acids (FA) arachidonic (AA) and docosahexaenoic (DHA) acids have been implicated in suboptimal infant growth, visual maturity and cognitive development.[1] From early gestation till term, the infant's brain weight increases from 20 to 378 g. At the same time, the lipid content of brain increases from 0.2 to 9.8 g at term, while the brain LCP content increases concomitantly.[2,3] Lipids account for 10% of adult wet brain weight, which corresponds to about 50% of dry matter.[4] These lipids are composed of glycerophospholipids (e.g. phosphatidylethanolamine, PE; and phosphatidycholine, PC) that are abundant in grey matter, and of sphingolipids (e.g. cerebrosides) which are notably located in white matter.[4]

During pregnancy, the fetus accretes substantial amounts of AA and DHA, which become selectively transported across the placenta by a process known as biomagnification.[5,6] Fetal tissues have the capacity to synthesize AA and to a lesser extent DHA[7] from their respective precursors; the essential FA (EFA) α-linolenic (ALA) and linoleic (LA) acids. The placenta, in contrast, is not considered to synthesize LCP.[7] Consequently, the fetal LCP status during pregnancy relates to the availability of EFA and LCP from the maternal diet and stores. Due to the small range of the maternal LCP status within Western countries, there are no studies that relate the maternal LCP status to the infant brain LCP contents at delivery or shortly thereafter. Postnatal studies indicated that infant tissue LCP contents are sensitive to dietary supply, since breastfed infants have higher levels of DHA in their brain, liver and adipose tissue compared to counterparts receiving formula or total parenteral nutrition devoid of LCP.[8-12] The higher LA content of infant formula, in turn, related to higher LA in brain,[8,9] liver[8] and adipose tissue.[10] The brain AA contents proved less sensitive to dietary deprivation and tended to increase in infants receiving formula or total parenteral nutrition without preformed DHA and AA,[8,9] although the liver[8,11] and adipose tissue[10] AA contents in these infants tended to be lower compared to controls receiving human milk.

Several studies[9,12,13] suggest that the infant's capacity to synthesize LCP from precursor is insufficient to reach brain DHA levels comparable to levels encountered in breastfed infants. However, the reference, i.e. Western human milk, in these studies might be questioned, since the current Western diet is relatively low in notably LCPω3 and high in LA, compared to our early ancestors' diet.[14] Moreover, the intake of LCPω3 by many Western populations does not comply with the recommendations that currently range from 250-450 mg/day,[15] which results in DHA-levels in Western human milk that are usually in the 0.3-0.4 g% range.[16,17] Consequently most Western infants develop in marginal intrauterine and postnatal LCPω3 environments with potentially adverse effects on brain development, although the latter proved difficult to attest in randomized controlled trials.[18-20] Also, there are no data that allow for comparison of fetal tissues of infants born to women with a low as compared to a high LCP status. It was recently shown, however, that baboons, fed with formula with much higher DHA (i.e. 1.00 g%) than the current milk of Western mothers, reached higher DHA levels in many organs and central nervous system regions, compared to controls that were fed with 0.33 g% DHA or no LCP from term birth to 12 weeks postpartum.[21] A similar experiment

in rhesus monkeys showed that a formula with 1% DHA and 1% AA caused earlier visual and motor abilities, compared to a formula with virtually no LCP.[22] Whether human infants from mothers with a milk DHA status of 1 g% give birth to infants with higher brain DHA levels may be expected, but is currently unknown, while no randomized controlled trials have focused on the neurodevelopmental aspects of such a high maternal DHA status during pregnancy, although one trial[23] has investigated the benefits of supplementing milk DHA levels around 1 g% on infant postnatal development.

During intrauterine development, the LCP become distributed among the various fetal organs. At term, the infant's brain contains the highest *relative* amounts of LCP,[3,9,13,24] but in an *absolute* sense most AA is located in the infants' adipose tissue (44%) and skeletal muscle (40%), with a third position for brain (11%), while DHA is notably in adipose tissue (50%) with second and third positions for brain (23%) and skeletal muscle (21%), respectively.[25] It has been suggested that at least part of the LCP in the infant's liver and adipose tissue can be mobilized and thereby have functions in postdelivery development.[26] This suggestion underscores the importance of an adequate maternal LCP status to support transplacental transport of polyunsaturated FA for both brain development and the development of infant LCP stores, as well as sufficient maternal LCP stores to support the subsequent postdelivery increase in infant DHA status, the predominant part of the fetal brain growth spurt and the continuing accretion of LCP in fetal storage organs. Finally, a high maternal LA status, such as typically observed in Western women with high intakes of vegetable oils, influences the ω3/ω6-balance by its inhibitory effect on ALA desaturation and elongation and by competition with LCP at the level of incorporation.[27] Taken together, we hypothesize that the high LCP and low LA intakes of African women with high fish consumption result in higher LCP and lower LA contents in fetal brain and storage organs, compared to infants born to Western women with low LCP and high LA status.

In the present study we studied the brain, liver and adipose tissue FA compositions in Tanzanian infants of various gestational ages who were stillborn to mothers with lifetime low intakes of LA from vegetable oils and very high intakes of both AA and DHA from the abundantly available local freshwater fish,[28] leading to mature milk DHA levels of about 0.63 g%.[29] The data were compared with the brain, liver and adipose tissue FA compositions of previously studied Western infants.[9-11,13,30] We were notably interested to link the outcome with our observation that at 10-20 weeks postpartum infants born to women from the same study area on the shores of Lake Victoria around Sengerema exhibited a more beneficial motor development than Dutch and other Tanzanian children with much lower fish intakes, which was explained by their higher DHA status after multivariate analysis.[31]

SUBJECTS AND METHODS

Data collection

Postmortem brain (n=18; gestational age (GA) 8-38 weeks), liver (n=14; GA 8-35) and adipose tissue (n=11; GA 19-38) samples were collected from 20 stillborn African fetuses and infants who were studied during a perinatal health project performed on the shorelines of Lake Victoria in Tanzania

in Sengerema Hospital. All infants were born to women with regular (average 4-5 times/week) fish intakes from the nearby lake.[28] Cases with any kind of neonatal problem that could affect cerebral integrity were excluded. Causes of death were obstructed labor or cord prolapse during delivery or premature death within 6 h after delivery due to immaturity, respiratory disease or infection. One premature infant died 1 day after delivery and had received a small amount of milk from its mother (infant no. 18); all others had received no oral or intravenous feeding after delivery. Since no ultrasound was available, gestational ages were approximated with aid of the measured body weight, crown-rump length (CRL) and head circumference (HC). Outcomes were compared with a reference database for these parameters in Australian infants,[32] since no such data were available for African infants. The study was approved by the National Institute for Medical Research in Dar-es-Salaam, Tanzania (NIMR/HQIR.8a/Vol. IX/145, dated June 16, 2003 and NIMR/HQ/R.8a/Vol. IX/800, dated April 8, 2009) and was in agreement with the Helsinki declaration of 1975 as revised in 2000.

Women who had miscarriages or delivered stillborn infants were asked permission to collect small (0.5-2 cm^3) samples of adipose tissue, liver and brain from their babies by needle biopsy. All included data derive from infants of whom their mothers gave informed consent. Samples were collected with 1 h after delivery and the locations of the biopsies were carefully sutured. After sampling, infants were returned to the mother. Adipose tissue samples were collected from the lower abdominal wall or the buttocks. Liver and brain samples were collected by direct biopsy from either the lower right abdominal wall or through the anterior fontanel. Brain samples were obtained by a 6 mm diameter specially designed cannula that could contain about 2 ml of brain, composed of a combination of grey and white matter.

Fatty acid analysis

Directly after biopsy, samples were transferred into a teflon-sealable Sovirel tube containing 2 mL of methanol/6 mol/L HCl (5:1 v/v), 1 mg butylated hydroxytoluene (antioxidant) and 50 µg of 17:0 as an internal standard.[33] All samples were transported at room temperature to the University Medical Center Groningen (The Netherlands) for FA analysis. Analyses of FA methyl esters were performed by capillary gas chromatography/flame ionization detection according to previously described procedures.[33] FA compositions were expressed in g%.

Statistics

Statistical analyses were performed with PSAW version 18.0 (SPSS Inc, Chicago, IL). Correlations were investigated using *Spearman's rho* for non-parametric regression. Plots of linear and curvilinear (inverse, exponential, polynomial) regression were tested for all FA. Plots that fitted the data best were selected.

RESULTS

Infant anthropometrics are shown in **Table 1**. Gestational ages derived from the infants' weight. The crown-rump length (CRL) and head circumference (HC) differed 3 weeks at most, but no more than 1 week in the majority (69%) of cases.

Table 1. Infant characteristics and estimated gestational age

	Sexe	Sexe	Weight	CRL	HC	BPD	GA	Samples collected		
	male=1	male=1	g	cm	cm	cm	weeks	brain	liver	adipose
Infant 1	?	?	<100	4			8	+	+	-
Infant 2	1	1	<100	8			12	+	+	-
Infant 3	1	1	<100	8			13	+	+	-
Infant 4	?	?	100	12		4	16	+	+	-
Infant 5	2	2	200	15			17	+	+	-
Infant 6	2	2	200	15		4	18	+	+	-
Infant 7	1	1	300	15			19	+	+	-
Infant 8	2	2	250	17			19	+	+	+
Infant 9	1	1	400	17	18	5	21	+	-	-
Infant 10	2	2	400	19			21	+	+	+
Infant 11	2	2	450	19	17	5	21	+	+	-
Infant 12	1	1	600	21			23	+	+	+
Infant 13	2	2	700	21		7	24	+	+	+
Infant 14	2	2	800	21	24	7	25	+	-	+
Infant 15	2	2	-	24			26	-	+	+
Infant 16	2	2	750	24	25	7	26	+	-	+
Infant 17	1	1	2200	30	30	8	34	+	-	+
Infant 18	1	1	2200	31	32		35	+	+	+
Infant 19	2	2	2600	32	33	8	37	+	-	+
Infant 20	1	1	3100	33	33	9	38	-	-	+

Abbreviations: CRL, crown rump length; HC, head circumference; BPD, biparietal diameter; GA, gestational age. The +/-, indicates whether (+) or not (-) a sample was collected

Brain, liver and adipose tissue FA composition during gestation

Data on the brain, liver and adipose tissue compositions (median and ranges) of the infants are shown in **Tables 2-4** and **Figures 1-3**. Although we performed a cross sectional study, several FA suggested significant changes with time.

Brain FA composition during gestation

In brain, the saturated 14:0 and 22:0 showed decreases with advancing gestation, but the net increase of 18:0 resulted in a net increase of saturated FA (SAFA) with length of gestation. Most individual monounsaturated FA (MUFA) and the sum of MUFA, except for 16:1ω7, decreased with length of gestation (Table 2; Figure 1A). Polyunsaturated FA (PUFA) (Figure 1A), including DHA (Figure 1B), 20:3ω6, 22:4ω6 and 22:5ω6 (Figure 1C), increased with advancing gestation, but AA, 20:3ω9 and 22:3ω9 all decreased (Figure 1B and 1D), while LA, which showed no change (Figure 1B).

Using the equations of Figure 1, we found that the LA content of brain remained constant at

Table 2. Brain tissue total lipid fatty acid composition

	n = 6	n = 9	n = 3	
Gestational age	14(8-18) wks	22(19-26) wks	35(34-37) wks	Spearman
14:0	2.22(1.88-3.09)	2.18(1.72-2.71)	1.58(1.53-1.87)	-0.590
16:0	30.4(28.5-31.0)	30.4(29.0-31.3)	30.1(26.8-31.8)	
18:0	18.5(14.6-19.3)	19.2(18.3-21.0)	20.9(18.4-22.3)	0.660
20:0	0.17(0.13-0.24)	0.15(0.13-0.19)	0.18(0.16-0.19)	
22:0	0.10(0.06-0.12)	0.07(0.05-0.11)	0.06(0.05-0.08)	-0.560
24:0	0.08(0.05-0.12)	0.06(0.03-0.18)	0.12(0.05-0.13)	
SAFA	50.7(48.4-52.3)	52.2(51.2-54.7)	52.4(51.0-52.9)	0.520
16:1ω7	2.06(1.49-3.94)	1.69(1.16-2.26)	1.75(1.44-4.33)	
18:1ω7	4.98(4.75-6.01)	4.65(3.76-5.08)	3.44(3.10-4.06)	-0.913
Σω7	7.41(6.29-9.63)	6.40(5.30-6.84)	5.57(5.24-7.47)	-0.645
18:1ω9	14.9(13.3-17.6)	14.0(12.4-14.5)	12.8(12.5-15.7)	-0.469
20:1ω9	0.70(0.63-1.02)	0.62(0.58-0.74)	0.54(0.44-0.62)	-0.722
20:3ω9	1.35(0.87-2.65)	0.92(0.77-1.37)	1.03(0.71-1.07)	-0.628
22:3ω9	1.40(1.00-2.87)	0.99(0.73-1.13)	0.80(0.62-1.20)	-0.724
24:1ω9	0.29(0.11-0.33)	0.18(0.15-0.30)	0.14(0.11-0.15)	-0.517
Σω9	19.6(16.1-23.9)	16.6(15.2-17.5)	15.9(15.0-17.5)	-0.620
MUFA	24.4(20.5-28.0)	20.9(18.9-22.3)	18.8(18.7-23.7)	-0.654
18:3ω3	0.00(0.00-0.02)	0.00(0.00-0.02)	0.01(0.00-0.01)	
20:5ω3	0.05(0.01-0.12)	0.05(0.02-0.10)	0.05(0.05-0.07)	
22:5ω3	0.24(0.16-0.56)	0.26(0.15-0.39)	0.24(0.21-0.31)	
22:6ω3	5.90(3.15-6.18)	6.78(5.21-7.59)	7.42(7.42-9.00)	0.832
ΣLCPω3	6.13(3.45-6.82)	7.08(5.40-7.95)	7.79(7.74-9.26)	0.817
Σω3	6.13(3.46-6.83)	7.08(5.41-7.96)	7.79(7.75-9.27)	0.817
18:2ω6	0.34(0.21-0.37)	0.34(0.26-0.75)	0.41(0.30-0.42)	
20:3ω6	0.35(0.20-0.40)	0.57(0.36-0.77)	0.81(0.75-0.96)	0.895
20:4ω6	10.3(9.55-11.09)	10.1(9.10-10.6)	8.81(7.37-9.10)	-0.480
22:4ω6	4.41(2.91-5.33)	4.62(3.86-4.93)	5.31(4.36-5.44)	0.550
22:5ω6	1.39(0.82-2.75)	2.38(1.77-2.95)	2.88(1.89-3.16)	0.527
ΣLCPω6	16.6(13.8-18.9)	17.4(16.4-18.5)	17.9(14.4-18.7)	
Σω6	16.9(14.1-19.3)	18.0(16.7-18.7)	18.2(14.9-19.2)	
LCPω3+LCPω6	22.4(17.7-25.0)	24.4(23.0-25.9)	25.6(22.1-28.0)	0.621
PUFA	25.6(23.6-27.2)	26.9(25.3-28.0)	28.3(23.9-30.3)	0.519

Abbreviations: wks, weeks; SAFA, saturated fatty acids (FA); MUFA, monounsaturated FA; LCP, long-chain polyunsaturated FA (≥20); PUFA, polyunsaturated FA. Data are expressed as median (range)

about 0.3-0.4 g% throughout gestation, while brain AA decreased from 11.1 g% (calculated; not shown) at 8 weeks to about 8.6 g% after 38 weeks and 8.4 g% after 40 weeks gestation (**Table 5**). Brain DHA increased from 3.2 g% (calculated, not shown) at 8 weeks gestation to 8.2 g% at 38 weeks and 8.4 g% at 40 weeks (Table 5).

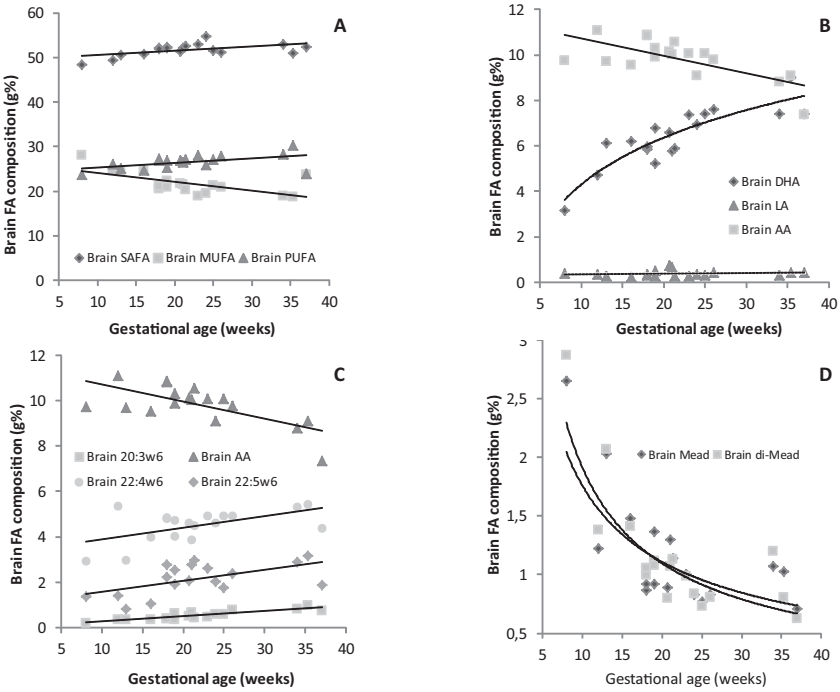

Figure 1A-D. Apparent courses of selected fatty acids in the brain of Tanzanian infants as a function of gestation. The infants were stillborn to mothers with lifetime low intakes of LA from vegetable oils and very high intakes of both AA and DHA from abundantly available local freshwater fish. One child of 35 weeks was life born and fed for 1-2 days. Data are in g/100g FA (g%) and weeks. Best fits were obtained by assuming linear, logarithmic, exponential and inverse relationships. As functions of gestational age (g% and weeks): Brain SAFA = 0.094x + 49.68 (R^2=0.27); MUFA = -0.196x + 26.00 (R^2=0.39); PUFA = 0.102x + 24.32 (R^2=0.23); DHA = 3.199ln(x) - 3.45 (R^2=0,70); AA = -0.083x + 11.75 (R^2=0.54); LA = 0.002x + 0.33 (R^2=0.01); 22:4ω6 = 0.061x + 3.40 (R^2=0.30); 22:5ω6 = 0.049x + 1.07 (R^2=0.32); 20:3ω6 = 0.023x + 0.024 (R^2=0.81); Mead acid (20:3ω9) = $8.206x^{-0.668}$ (R^2=0.57); Di-Mead acid (22:3ω9) = $12.299x^{-0.807}$ (R^2=0.70).

Liver FA composition during gestation

In the liver, SAFA increased significantly, notably because of 18:0. MUFA decreased with length of gestation, notably on account of 18:1ω7 (Table 3; Figure 2A). Similarly, both Mead (20:3ω9) and di-Mead acid (22:3ω9) decreased (Table 3), while PUFA, LA, 20:3ω6 and 22:4ω6 increased with advancing gestation (Table 3; Figure 2A and 2B). These increases occurred regardless of the inclusion of the 35 weeks old infant who had received oral feeding. Conversely, after exclusion of the 35 weeks old infant, DHA and AA showed no significant changes during gestation (Figure 2A and 2B), although AA decreased when the 35 weeks old infant was included (dotted line, $p<0.001$).

Liver LA increased from 0.72 g% at 8 weeks to 9.6 g% at 35 weeks gestation, while liver AA showed no change from early gestation till about 27 weeks gestation (Figure 2). However, if the low liver AA content of the 35 weeks infant with feeding was included into the calculation (Figure 2B), AA decreased substantially from 13.6 g% at 8 weeks (calculated, not shown) to 7.2 g% at 35 weeks

Table 3. Liver tissue total fatty acid composition

	n = 6	n = 7	n = 1	
Gestational age	14(8-18) wks	22(19-26) wks	35 wks	Spearman
14:0	0.51(0.40-0.76)	0.48(0.36-0.59)	1.30	
16:0	23.9(20.5-24.4)	24.8(23.3-27.5)	35.3	
18:0	14.8(13.7-17.2)	16.8(16.1-18.2)	9.56	0.592
20:0	0.29(0.26-0.33)	0.38(0.32-0.42)	0.16	0.675
22:0	0.60(0.49-0.68)	0.70(0.66-1.03)	0.32	0.843
24:0	0.92(0.82-1.01)	0.97(0.83-1.30)	0.52	
SAFA	40.7(38.4-43.5)	44.4(42.4-46.9)	47.2	0.700
16:1ω7	3.52(2.02-4.51)	2.17(1.47-2.90)	5.87	
18:1ω7	5.61(4.17-6.58)	3.96(3.65-4.14)	3.40	-0.085
Σω7	9.22(6.25-10.9)	6.31(5.69-6.62)	9.29	-0.590
18:1ω9	18.8(16.5-25.5)	16.6(9.66-20.3)	19.3	
20:1ω9	0.29(0.23-0.36)	0.27(0.10-0.38)	0.12	
20:3ω9	1.47(1.04-3.89)	0.98(0.67-1.39)	0.24	-0.518
22:3ω9	0.20(0.15-0.45)	0.16(0.10-0.21)	0.00	-0.573
24:1ω9	1.66(1.41-2.05)	1.75(1.12-2.42)	0.33	
Σω9	23.7(20.3-28.9)	20.0(12.4-23.4)	20.0	-0.501
MUFA	30.3(26.0-36.7)	25.2(17.5-28.4)	29.0	-0.658
18:3ω3	0.02(0.01-0.05)	0.04(0.02-0.07)	0.17	
20:5ω3	0.37(0.08-1.34)	0.31(0.15-0.66)	0.12	
22:5ω3	0.21(0.09-0.45)	0.23(0.12-0.38)	0.30	
22:6ω3	4.68(3.73-6.59)	4.92(3.43-6.68)	4.59	
ΣLCPω3	5.22(3.91-8.39)	5.44(3.72-7.63)	5.02	
Σω3	5.24(3.94-8.44)	5.48(3.76-7.66)	5.19	
18:2ω6	2.27(1.20-4.26)	5.13(3.61-5.75)	9.79	0.736
20:3ω6	1.19(0.84-1.46)	1.74(1.45-2.28)	0.75	0.793
20:4ω6	16.0(12.9-18.9)	16.9(14.9-17.9)	6.65	
22:4ω6	0.56(0.40-0.89)	0.69(0.56-1.12)	0.36	0.559
22:5ω6	0.68(0.53-1.21)	0.60(0.42-0.92)	0.56	
ΣLCPω6	18.7(15.4-21.5)	20.1(17.9-21.2)	8.47	
Σω6	20.7(16.8-25.2)	25.0(22.3-26.4)	18.4	
LCPω3+LCPω6	25.1(19.6-26.8)	25.4(21.6-28.7)	13.5	
PUFA	30.0(23.9-30.5)	30.6(28.1-35.5)	23.8	0.642

Data are expressed as median (range). For abbreviations: see Table 2. Due to the remarkable differences compared to the younger infants, liver FA compositions from the 35 wks old infant were not included for regression analysis, since th infant had received a small amount of milk of unknown origin from its mother shortly after delivery.

gestation (calculated, not shown). In this 35 weeks old infant the substantially lower liver AA (6.7 g%) was accompanied by higher 16:0, 16:1ω7; 18:1ω9 and LA, compared to the other infants, but its DHA content was similar. We found that liver DHA remained constant at about 4.7-4.9 g% during gestation (**Table 6**).

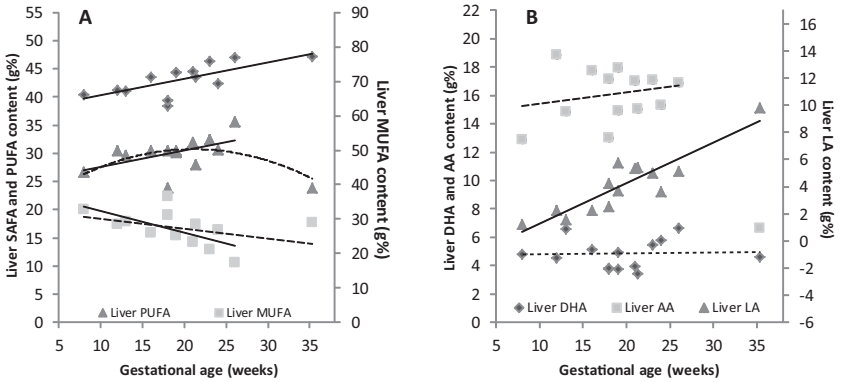

Figure 2A-B. Apparent courses of selected fatty acids in the liver of Tanzanian infants as a function of gestation. Insignificant relations according to Spearman's rho are indicated by dotted lines. For further legend see Figure 1. As functions of gestational age (g% and weeks): Liver SAFA = 0.293x + 37.37 (R^2=0.50); MUFA (35 weeks infant included) = -0.282x + 32.79 (R^2=0.15); MUFA (35 weeks infant excluded) = -0.62x + 38.51 (R^2=0.40); PUFA (35 weeks infant included) = -0.025x^2 + 1.093x+19.22 (R^2=0.29); PUFA (35 weeks infant excluded) = 0.295x + 24.65 (R^2=0.29); DHA = 0.008x + 4.65 (R^2=0.002); LA = 0.299x - 1.72 (R^2=0.80); AA (35 weeks infant included) = -0.032x^2 + 1.137x+6.59 (not shown; R^2=0.67); AA (35 weeks infant excluded) = 0.08x + 14.60 (R^2=0.05).

Adipose tissue FA composition during gestation

In adipose tissue, the net increase of 14:0 and 16:0 (Table 4) explained the net increase of SAFA with length of gestation (Table 4, Figure 3A), despite the concurrent decreases of 22:0 and 24:0. The substantial increase in adipose tissue SAFA towards the pregnancy end coincided with decreases of MUFA, PUFA, DHA, LA and AA (Figures 3A-D) with advancing gestation.

We could not locate any adipose tissue in infants younger than 19 weeks. Using the equations from Figure 3, we calculated that LA decreased from about 3.2 g% at 19 weeks gestation to 0.92 g% at 38 weeks, while AA decreased from about 3.9 g% at 19 weeks to 0.42 g% at 38 weeks. DHA decreased from 3.8 g% at 19 weeks gestation to 0.43 g% at 38 weeks gestation (**Table 7**).

DISCUSSION

We measured the brain, liver and adipose tissue FA compositions of African infants who were stillborn to mothers with lifetime low intakes of LA from vegetable oils and very high intakes of both AA and DHA from abundantly available local freshwater fish. Our data show that with increasing gestation brain DHA increases, while AA decreases as a percentage of total FA in brain. In contrast to AA, all other LCPω6 exhibited concurrent increases, while both 20:3ω9 and 22:3ω9 showed substantial decreases from early gestation until term. In the fetal liver, we found no significant changes of DHA with length of gestation, but LA increased significantly. AA remained constant from early gestation until about 30 weeks, where after we measured one very low liver AA content in a single infant. In adipose tissue, a significant increase in SAFA coincided with net decreases of LA, AA and DHA with advancing gestation.

Table 4. Adipose tissue total fatty acid composition

	n = 6	n = 5	
Gestational age	23(19-26)	36(34-38)	Spearman
14:0	2.62(2.05-3.57)	3.63(3.26-4.12)	0.797
16:0	34.9(31.1-42.6)	48.2(47.5-52.3)	0.980
18:0	3.80(3.21-8.79)	6.19(4.52-7.11)	
20:0	0.06(0.04-0.19)	0.08(0.07-0.11)	
22:0	0.05(0.02-0.26)	0.01(0.01-0.01)	-0.961
24:0	0.10(0.03-0.45)	0.03(0.02-0.03)	-0.961
SAFA	43.1(38.6-49.5)	58.2(56.7-62.3)	0.952
16:1ω7	12.0(6.91-16.2)	10.2(9.39-13.1)	
18:1ω7	3.28(2.35-3.90)	1.71(1.45-2.37)	-0.847
Σω7	15.6(10.9-18.6)	11.9(10.9-15.6)	
18:1ω9	30.8(27.9-35.7)	25.3(23.1-28.8)	-0.665
20:1ω9	0.20(0.08-0.39)	0.07(0.07-0.14)	-0.861
20:3ω9	0.31(0.12-0.51)	0.09(0.07-0.14)	-0.884
22:3ω9	0.05(0.00-0.17)	0.00(0.00-0.00)	-0.847
24:1ω9	0.10(0.03-0.60)	0.01(0.01-0.02)	-0.925
Σω9	31.3(28.6-36.3)	25.4(23.4-29.0)	-0.838
MUFA	47.0(39.9-52.2)	38.8(35.8-39.7)	-0.765
18:3ω3	0.06(0.02-0.13)	0.01(0.01-0.05)	-0.765
20:5ω3	0.15(0.04-0.20)	0.03(0.02-0.04)	-0.866
22:5ω3	0.21(0.06-0.35)	0.06(0.04-0.06)	-0.829
22:6ω3	2.38(0.88-3.46)	0.49(0.42-0.54)	-0.834
ΣLCPω3	2.72(0.98-4.02)	0.56(0.52-0.63)	-0.834
Σω3	2.77(1.00-4.09)	0.59(0.53-0.64)	-0.852
18:2ω6	2.48(1.03-4.52)	0.96(0.74-2.23)	-0.765
20:3ω6	0.25(0.09-0.82)	0.07(0.05-0.10)	-0.911
20:4ω6	2.23(0.82-6.17)	0.56(0.36-0.84)	-0.888
22:4ω6	0.39(0.14-1.36)	0.11(0.07-0.18)	-0.879
22:5ω6	0.32(0.17-0.68)	0.08(0.05-0.20)	-0.879
ΣLCPω6	3.24(1.28-9.22)	0.84(0.58-1.40)	-0.843
Σω6	5.88(2.38-13.9)	1.85(1.35-3.73)	-0.888
LCPω3+LCPω6	6.14(2.26-11.6)	1.42(1.10-1.99)	-0.943
PUFA	9.05(3.52-17.1)	2.53(1.97-4.50)	-0.897

Data are expressed as median (range). For abbreviations: see Table 2.

Infant brain FA composition

Our data for the brain FA composition of African infants with duration of gestation (Figure 1) are in line with earlier reports, showing an increase of DHA and a decrease of AA in infant brain PE with advancing gestation.[3,8] Unfortunately, direct comparison of the present data with most of these earlier reports is virtually impossible, since these authors reported FA compositions of the separate PL species in brain,[3,8] while another difference is the separation of cerebral white and grey matter.[24] Nevertheless, our present findings suggest that the net effect of the sometimes opposing courses of

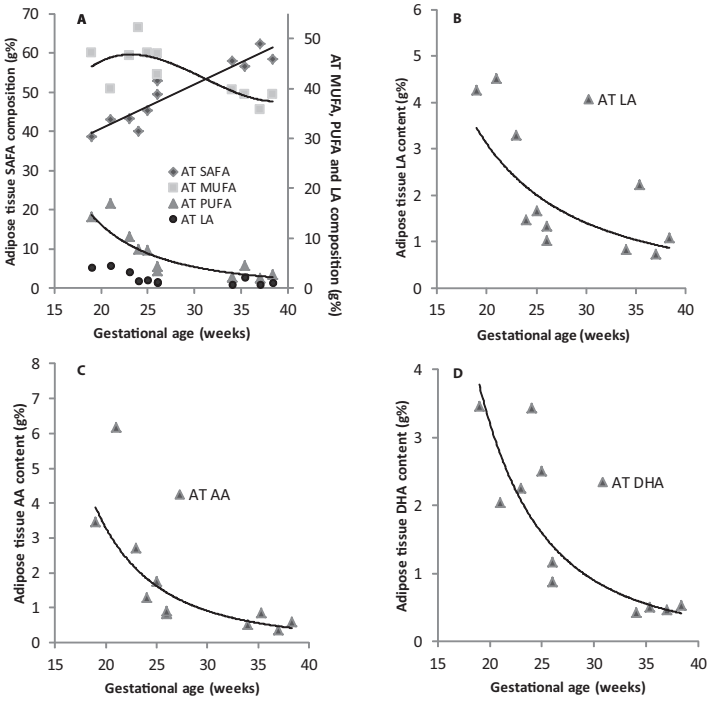

Figure 3A-D. Apparent courses of selected fatty acids in adipose tissue (AT) of Tanzanian infants as a function of gestation. For further legend see Figure 1. As functions of gestational age (g% and weeks): Adipose tissue: SAFA = $1.003x + 22.24$ (R^2=0.81); MUFA = $0.012x^3 - 1.068x^2 + 31.237x - 249.13$ (R^2=0.62); PUFA = $73138x^{-2.867}$ (R^2=0.84); LA = $616.17x^{-1.79}$ (R^2=0.56); AA = $43965x^{-3.172}$ (R^2=0.77); DHA = $39197x^{-3.141}$ (R^2=0.83).

AA and DHA in the different PL species in white and grey matter[24] results into a net increase of DHA and net decrease of AA with advancing gestation, and that at term age brain DHA levels are equal to those of AA (Figure 1B).

The increasing similarity of infant brain DHA and AA levels in a term infant supports, to a certain extent, observations of others who studied the infant whole brain FA composition at term and shortly after delivery in relation to the infants' diet.[8,9,13] Although, to our knowledge no hard data were published for the FA compositions (in g%) of the preterm or term infant brains prior to the initiation of feeding, one of the several studies by Martinez[30] provides data that allow for the calculation* of the brain LCP composition of preterm (GA 26-36 weeks) and term (GA 37-42 weeks) Spanish infants. Two additional studies provide data on the FA composition of the infant brain in relation to breast or formula feeding at several weeks postpartum.[9,13] Data of these studies and the present are presented in Table 5. A comparison suggests that the presently calculated brain contents of 8.2 g% DHA at 38 weeks GA in these African infants is higher compared to that of Spanish stillborn preterm infants (GA 26-36 weeks, 7.3 g% DHA) and stillborn term infants (GA 37-42 weeks, 7.8 g% DHA).

* Using a molecular weight (MW) of 292 mol/g for total FA and MWs of 326, 304, 332 and 328 for DHA, AA, 22:4ω6 and 22:5ω6, respectively.

Table 5. The brain DHA, AA, 22:4ω6 and 22:5ω6 composition and DHA/AA ratio in the current study compared to the literature

	Kuipers whole brain					Martinez[a] whole brain		Farquharson[b] cortex grey matter		Makrides[c] cortex white and grey matter		Farquharson[b] cortex grey matter	
	very preterm 8-18 wks stillborn no feeding	preterm 19-26 wks stillborn no feeding	preterm 34-37 wks stillborn no feeding	term[d] 38 wks calculated no feeding	term[d] 40 wks calculated no feeding	preterm 26-36 wks stillborn no feeding	term 37-42 wks stillborn no feeding	term 9 wk PP lifeborn breastfed	term 9 wk PP lifeborn formula fed	term 16 wk PP lifeborn breastfed	term 17 wk PP lifeborn formula fed	term 23 wk PP lifeborn formula fed	term 22 wk PP lifeborn formula fed
	n=6	*n=9*	*n=3*	*n=18[d]*	*n=18[d]*	*n=8*	*n=13*	*n=5*	*n=5*	*n=15*	*n=20*	*n=5*	*n=5*
DHA	5.9	6.8	7.4	8.2	8.4	7.3	7.8	9.7	7.6	8.5	7.5	7.5	6.6
AA	10.3	10.1	8.8	8.6	8.4	10.2	9.2	10.6	12.2	10.9	11.2	12.9	12.6
22:4ω6	4.4	4.6	5.3	5.7	5.8	5.8	6.0	5.8	6.7	6.6	7.0	6.8	6.9
22:5ω6	1.4	2.4	2.9	2.9	3.0	3.2	2.7	2.2	3.3	3.0	3.5	3.1	4.2
DHA/AA	0.57	0.67	0.84	0.95	1.00	0.72	0.85	0.92	0.62	0.78	0.67	0.58	0.52

Abbreviations: DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; wk(s), week(s). Data are presented as means (g/100 g fatty acids (g%); and g/g fatty acids). [a], adapted from Martinez (30); [b], adapted from Farquharson et al.[9,c]; adapted from Makrides et al.[13a, d]; Data were calculated using the equations from Figure 1: based on 18 samples.

Since brain DHA is known to increase rapidly after birth,[3,8] it is inappropriate to compare with postnatal data. However, whole brain DHA in the term stillborn African infants seems higher compared to the cortex white and grey matter DHA content of formula fed infants (6.6-7.6 g% DHA).[9,13]

In contrast, brain AA, 22:4ω6 and 22:5ω6 seem lower in the African infants compared to Western counterparts (Table 5). The presently found brain content of 8.6-8.4 g% AA at term age in our African infants is lower compared to the 9.2 g% AA in the Spanish term infants,[30] while despite the postpartum decrease in brain AA, the older English[9] and Australian[13] infants had even higher brain AA contents (breastfed 10.6-10.9; formula-fed 11.2-12.9 g% AA) between 9-23 weeks postpartum. A similar picture emerged for 22:4ω6 and 22:5ω6. Consequently the DHA/AA ratio in the African infant at term age (0.95-1.0 g/g) was higher compared to that of breastfed and formula fed Western infants (0.52-0.92 g/g).

The rate of LCP synthesis in the adult vertebrate brain is insignificant.[34] However, no such data are, as yet, available for the human fetal brain.[34] Stable isotope studies showed that although ALA becomes primarily recovered as 22:5ω3 and DHA in the neonatal baboon brain, dietary DHA is 7 times more effective than dietary ALA as a source for brain DHA accretion.[35] In contrast, it was estimated that about

Table 6. Liver DHA, LA and AA composition and the DHA/AA ratio in the current study and the literature

	Kuipers liver			Martinez[a] liver		Farquharson[b] liver	
	very preterm 8-18 wks stillborn no feeding	preterm 19-26 wks stillborn no feeding	preterm 35 wks stillborn milk fed	preterm 25-30 wks stillborn no feeding	term full term stillborn no feeding	term 1 wk PP calculated breastfed	term 10 wk PP calculated breastfed
	n=6	*n=7*	*n=1*	*n=2*	*n=3*	*n=7*	*n=7*
DHA	4.7	4.9	4.6	3.7	4.1	5.8	3.7
LA	2.3	5.1	9.8	6.3	7.9	-	-
AA	16.0	16.9	6.7	12.8	8.6	8.4	6.1
DHA/AA	0.29	0.29	0.69	0.29	0.48	0.69	0.61

Abbreviations: DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; wks, weeks. Data are presented as means (g/100 g fatty acids (g%); and g/g fatty acids). [a], Adapted from Martinez[30];
[b], Calculated from the equations in Farquharson et al.[11]; which are based on a trendline fited to a total of 7 sampled infants ranging from 1 to 22 weeks postpartum.

50% of brain AA derives from dietary LA, while about 20% of brain 22:4ω6 and 10% of 22:5ω6 derive from dietary LA.[35,36] Taken together, it is assumed that LCP in the mammalian fetal brain derive either directly from the maternal circulation or from desaturation/elongation of ALA and LA in the fetal liver. With regard to the latter, it is was shown that in adult rats fed an ω3PUFA diet devoid of DHA, liver synthesis of DHA proved sufficient to maintain brain DHA levels.[34] Subsequent studies with LCP[21,36,37] and PUFA deprived diets suggested that the contents of both DHA and AA in the central nervous system are strictly regulated, but sensitive to dietary intakes. That is, ω3-PUFA deprived diets reduce DHA-turnover, while reciprocally increasing AA-metabolism[38] and promoting accumulation of 22:5ω6 in brain,[34] while ω6-PUFA deprived diets downregulate AA turnover[39] and upregulate DHA-metabolism.[39] In summary, the vertebrate brain has limited capacity to synthesize LCP from precursors, LCP from the circulation are rapidly taken up into the fetal brain, and multiple mechanisms support a certain equilibrium between LCP from the ω3- and ω6-series in brain tissue. In contrast to the above studies that describe observations made under severe LCP deprivation, the current results suggest that a high intake of fish increases fetal brain DHA and reduces brain AA, perhaps because LA metabolism becomes more directed at 20:3ω6, while AA becomes converted to its longer-chain storage forms, notably 22:4ω6 (Figure 1C).[36] It might in this respect be of importance to note that 20:3ω6 is the precursor of the series-1 prostaglandins, suggesting that major, dietary-induced, changes might occur in the functional balance between the highly active metabolites of 20:3ω6, AA, EPA and DHA[40,41] with as yet unknown consequences.

These combined observations support the existence of a bell-shaped relation between DHA and AA that is synergistic at low and antagonistic at high DHA or AA status.[42,43] Moreover, at high intakes of both AA and DHA, the brain AA content seems secondary to the available DHA for incorporation. A similar DHA vs. AA competition was previously noted in other compartments, notably erythrocytes and umbilical vessel walls.[43] At low DHA status and insufficient dietary ALA, brain AA levels become

Table 7. The adipose tissue DHA, LA and AA composition and the DHA/AA ratio in the current study compared to the literature

	Kuipers (Africa) adipose tissue				Kuipers (Curacao)[a] adipose tissue			Farquharson[b] adipose tissue		
	preterm 19-26 wks stillborn no feeding	preterm 34-38 wks stillborn no feeding	preterm[c] 19 wks calculated no feeding	term[c] 38 wks calculated no feeding	preterm 22-29 wks stillborn no feeding	preterm 29-37 wks stillborn no feeding	term 37-43 wks stillborn no feeding	term delivery lifeborn no feeding	term 11 wk PP lifeborn breastfed	term 17 wk PP lifeborn breastfed
	n=6	n=5	n=11	n=11	n=15	n=11	n=17	n=4	n=7	n=15
DHA	2.38	0.49	3.8	0.43	1.18	0.39	0.30	0.4	0.1	0.0
LA	2.48	0.96	3.2	0.92	5.90	2.97	2.76	2.2	3.6	13.7
AA	2.23	0.56	3.9	0.42	2.06	0.88	0.66	0.7	0.3	0.1
DHA/AA	1.07	0.88	0.97	1.02	0.57	0.44	0.45	0.57	0.33	0.00

Abbreviations: DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; wks, weeks. Data are presented as means (g/100 g fatty acids (g%); and g/g fatty acids). [a], Adapted from Kuipers et al.[47]; [b], Adapted from Farquharson et al.[10]; [c], Data were calculated using equations from Figure 3: based on 11 samples.

reduced, while increasing amounts of AA become deposited as 22:4ω6 and 22:5ω6;[34,38] while at low AA levels and insufficient dietary LA, brain DHA levels are reduced[39] by some other mechanism since the amounts of 20:5ω3 and 22:5ω3 in brain remain low.[35] On the other hand, our results now indicate that at high intakes of both DHA and AA, increasing brain DHA levels result in a decrease of brain AA, while increasing amounts of ω6PUFA become deposited as 20:3ω6, 22:4ω6 and 22:5ω6 (Figure 2C), but 20:5ω3 and 22:5ω3 again remain low and virtually unchanged. The concomitant decreases of 20:3ω9 and 22:3ω9 with advancing gestation (Figure 2D) suggest dilution by SAFA, MUFA and PUFA rather than a decreasing desaturation/elongation activity *per se*. More importantly, however, the nevertheless increasing relative brain DHA content supports the notion that dietary DHA is much more effective than its precursors as a source for brain DHA accretion.[35] Finally, high intakes of LA, although sometimes recommended,[44] might interfere with the desaturation/elongation of ALA, 20:5ω3 and 22:5ω3 to DHA.[27] As such, the high dietary intakes of LA in Western countries might not only directly increase the brain AA content (secondary to the conversion of LA to AA in the liver and the subsequent uptake of AA in the brain from the circulation), but high LA levels also increasingly compete with ALA for desaturation, resulting in lowers levels of circulating DHA that are made available for uptake in the brain.

Taken together, we found an increase in brain PUFA, DHA, 20:3ω6, 22:4ω6 and 22:5ω6 (and SAFA) at the expense of AA and MUFA

with advancing gestation. The African infants near lake Victoria had higher DHA and lower AA in brain than Western counterparts, which clearly relates to the lifetime high intake of DHA-rich, but also AA-rich, fish by their mothers. We recently found that the high erythrocyte DHA status of 10-20 weeks old infants living in the same geographical area is related to slightly more optimal motor development.[31] Although erythrocyte and brain DHA contents are intimately related,[45] the current study provides more direct evidence that the observed more beneficial motor development in these infants is indeed associated with higher amounts of DHA in their brains, such as in regions like the sulcus precentralis,[21,37] that is involved in motor coordination and integration of visual signals, among others.

Infant liver FA composition

In contrast to the constancy of liver DHA in our infants with advancing gestation (Figure 2), Martinez and Ballabriga[8] showed increases in DHA in PE and PC. Recalculation[1] of their other data[30] also revealed that as a percentage of total FA, liver DHA increased from 3.7 g% to 4.1 g% between 25-30 weeks gestation and full term age (Table 6). Recalculation** of the data from Farquharson et al.[11] shows that from 1 to 10 weeks postpartum, liver DHA decreased from 5.8 g% to 3.7 g% in breastfed infants (Table 6). Thus, compared to Western counterparts, our African infants seem to have somewhat higher liver DHA throughout pregnancy (4.6 g%; Table 6). Further comparison of the liver DHA content at term or postpartum seems inappropriate, since we as well as Farquharson et al.[11] included only a single infant at 35 weeks and 1 week postpartum, respectively.

Similar to liver DHA, we observed no significant changes in liver AA in early (<30 weeks) gestation (16.0-16.9 g%), while Martinez and Ballabriga[8] showed decreases of AA in liver PE and PC from early pregnancy (20-25 weeks) until term age (>36 weeks). Recalculation of their data for total FA showed that AA decreased from 12.8 g% to 8.6 g% (Table 6) between 25-30 weeks gestation and full term age. Similarly, Farquharson[11] showed that total hepatic AA decreased from 8.4 to 6.1 g% between delivery and 10 weeks postpartum (Table 6). Thus, the liver AA content of the (preterm) African infants upto about 30 weeks gestation seems considerably higher compared to those reported for Western infants.[8,11] Conversely, the infant who was born after 35 weeks gestation and who was fed for 1-2 days before death had a remarkably lower liver AA content (Figure 2) that was accompanied by a much higher LA content, suggesting that the milk that was fed increased LA and reduced AA in the liver. The postpartum increase in infant LA status, is indeed supported by the bioattenuation of LA during pregnancy, in contrast to the postpartum LA surge via the milk.[46]

Infant adipose tissue FA composition

Our data for the adipose tissue FA composition of African infants (Figure 3) are in line with earlier data from Farquharson[10] and our earlier report on adipose tissue of infants born to African-Caribbean mothers living in the island of Curaçao[47] (Table 7). With advancing gestation the adipose

** Calculated from the amount of DHA and AA in g/kg liver, using a liver lipid content of 50.3 g lipid/kg liver

tissue FA composition exhibits dramatic decreases of both PUFA and LCP. This occurs secondary to the increases of MUFA, and notably SAFA, giving rise to a dilution of PUFA, and eventually also of MUFA by the *de novo* synthesized SAFA. For instance, the adipose tissue AA content of the African infants decreased from a maximum of 6.2 g% at 19 weeks to a minimum of 0.36 g% at 40 weeks gestation. These data support our earlier finding that the extremely high AA (7.6 g%) and DHA (1.63 g%) contents as reported for infant adipose tissue at 22-43 weeks[48] overestimate the actual adipose tissue LCP contents. These erroneous data, however, have been frequently used as a reference for term adipose tissue.[7,49-51]

Comparison of the African fetal adipose tissue FA contents with those of Western counterparts revealed that term African infants have comparable or slightly higher adipose tissue DHA, but substantially lower LA and AA, resulting in an about 2 times higher DHA/AA ratio (Table 7). Again, the higher adipose tissue DHA contents likely results from the high maternal intakes of freshwater fish from the nearby Lake Victoria. An even higher adipose tissue DHA content in these infants may have become prevented by 'DHA bioattenuation',[52] which is an as yet poorly understood process causing lower DHA in the fetal circulation as compared with the maternal circulation at high maternal DHA status, as opposed to the 'biomagnification' at low DHA status.[5] The low adipose tissue LA content is in line with their previously noted low intakes of LA. The latter becomes translated into the low LA in milk[53] and erythrocytes[46] that are observed in several non-Westernized Tanzanian populations. Interestingly, the low LA contents of breast milk of women living in the Tanzanian islands of Chole (4.23 g% LA) and Ukerewe (5.20 g% LA), was even lower compared to current recommendations for the intake of LA from infant formulas.[53] The ensuing higher adipose tissue LA content of Western infants has already been noted in 1975.[54] The by then high adipose tissue LA content of Dutch (2.9 g%) compared to British (1.0 g%) infants at delivery was attributed to the high maternal intakes of LA-rich soft margarines in the Netherlands, while the much higher LA in body fat of 4 months old breastfed Dutch (32-37 g% g%), compared to British (3-4 g%) infants, was attributed to the addition of LA to cow's milk in the Netherlands, as opposed to the addition of carbohydrates in Britain.[54]

The high adipose tissue and milk LA contents of Western compared to some African countries has been implicated in the origin of typically Western diseases.[55,56] Clearly, milk has to provide sufficient LA for the synthesis of LA-rich ceramides that constitute the skin-water barrier[57,58] and also for the synthesis of AA for the brain and other organs. However, the latter reaches maximum capacity from 4 energy% LA.[35] The enormous surplus of LA provided with Western milks contrasts with the low amounts of LA in milk during human evolution, as e.g. supported by the observations of traditionally living African tribes[53] who do not consume high amounts of (refined) vegetable oils. The question whether the resulting composition of body fat matters, has also been posed before,[54] but remains unresolved as yet. A recent meta-analysis from LA-intervention studies revealed that higher LA intakes by adults produced no indication of benefit but rather a fairly consistent, but non-significant, signal toward *increased* risk of coronary heart disease and death.[56] These data underscore the possible adverse effects of the high, and still increasing, intakes of LA in Westernized societies.[55,56]

Secondly, the higher adipose tissue LA contents in infants, coincided with lower amounts of SAFA,[54] notably medium-chain-SAFA (MCSAFA). It has been shown earlier, that both dietary lauric (12:0) and myristic (14:0) acids can be stored in adipose tissue.[59] However, while the adipose tissue LA increases continuously after delivery, MCSAFA show a steep postpartum increase that is followed by a decrease after the discontinuation of lactation, suggesting that MCSAFA in adipose tissue are more readily available for energy generation or other metabolic purposes. Indeed, with its long half-life of 680 days,[60] LA has been suggested to play a role in the development of adiposity.[55,61] Conversely, MCSAFA have been associated with downregulation of key adipogenic genes such as peroxisome proliferator activated receptor-γ (PPAR-γ), reduced adipose tissue lipoprotein lipase activity, reduced fat pads and improved insulin sensitivity in rats fed a high-MCSAFA diet, compared to counterparts receiving a high-LA diet.[62]

A final concern derives from the well known inhibitory effects of high LA intakes on FA desaturase (FADS)-activities. In their classical study, Mohrhauer and Holman[63] showed that a diet high in ALA, resulted in low tissue AA levels. Conversely, a diet high in LA resulted in low tissue DHA levels.[63] It was also shown[64] that at constant high LA intakes, AA synthesis is adjusted to concurrent AA intakes. Dietary LA intakes also correlate inversely with both DHA and AA in tissue.[65] At the already high LA intakes in Western countries, any further increases in LA intake is unlikely to additionally increase the, already maximized, AA status. However, the high LA intake by Western women might, secondary to their concomitantly low DHA intakes, additionally compromise their DHA status and tissue ω3/ω6 ratio by direct competition for incorporation of LA and AA with DHA and also by negative feedback of AA on FADS-activity. In contrast to Western counterparts, postnatal African infants have low LA intakes, but high intakes of both preformed AA and DHA from their mother's milk, which, in turn, derives from the frequent intake of AA and DHA-rich local freshwater fish.

CONCLUSIONS

The present study supports previous FA data for brain, liver and adipose tissue, showing comparable FA changes with advancing gestation. That is, with advancing gestation, brain SAFA, PUFA, DHA and 20:3ω6, 22:4ω6 and 22:5ω6 increase, while MUFA, 20:3ω9, 22:3ω9 and AA decrease. The decrease in brain AA might be secondary to an augmented AA-metabolism that results in increases of 20:3ω6 (the precursor of the series-1 prostaglandins), 22:4ω6 (the main storage form of AA in the brain) and 22:5ω6. In the liver, SAFA, PUFA and LA increase, while MUFA decreases with gestation. The steep increase of (mostly *de novo* synthesized) SAFA in adipose tissue causes relative decreases of MUFA, PUFA, DHA, LA and AA with advancing gestation. Compared to Western infants, the currently studied African infants, born to mothers with lifetime low intakes of LA from vegetable oils and very high intakes of both AA and DHA from abundantly available local freshwater fish, had higher DHA, but lower AA, and consequently a higher DHA/AA-ratio in brain; while also their adipose tissue was higher in DHA, but lower in LA and AA. We suggest that the low LA intake and the high DHA and AA intakes of African women living close to lake Victoria might support optimal ALA vs. LA

competition for Δ5D and Δ6D-activities and DHA vs. AA antagonism. Conversely, the Western diet, which is characterized by much higher intakes of LA and lower DHA and AA consumption, might disturb these evolutionary conserved mechanisms aiming at an optimal ω3/ω6-balance.

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CHAPTER 6.1

Gestational age dependent content, composition and intrauterine accretion rates of fatty acids in fetal white adipose tissue

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ABSTRACT

Background. Little is known about the gestational age (GA) dependent content, composition and intrauterine accretion rates of fatty acids (FA) in fetal white adipose tissue (WAT).

Objective & design. To acquire this information, we collected abdominal subcutaneous WAT samples from 40 preterm and term fetuses. Their GA ranged from 22-43 weeks. FA were expressed as mg/g wet WAT and g/100 g FA (g%). Intrauterine WAT FA accretion rates were estimated for appropriate (AGA) and large (LGA) for gestational age infants.

Results. From 25-40 weeks gestation, saturated-FA (SAFA) increased from 83-298 mg/g WAT and monounsaturated-FA (MUFA) from 83-226 mg/g WAT, while polyunsaturated-FA (PUFA) increased insignificantly from 18.0-23.2 mg/g WAT. As percentages of total FA, SAFA increased from 46-55 g%, MUFA decreased from 44-41 g%, and PUFA from 10.3-4.26 g%. Docosahexaenoic (DHA) and arachidonic acid (AA) accretion rates in WAT during the 3rd trimester for AGA infants were 88 and 193 mg/week, respectively. Contemporaneous DHA and AA accretion rates for 4,500 g LGA infants were 184 and 402 mg/week, respectively. Compared to the whole 3rd trimester, increment rates during the last 5 weeks of gestation were about 2-fold higher.

Conclusion. FA accretion rates, notably those of DHA and AA, may be important for designing nutritional regiments for preterm infants. The current WAT-DHA and WAT-AA accretion rates are considerably lower than previously reported in the literature.

INTRODUCTION

Most intrauterine changes in the fetal body composition have been studied in great detail. For instance, from 12 weeks gestation to term, body water decreases from 92-70 g%, total body protein increases from 5.5-12 g% and total body lipid increases from 0.5-16 g%.[1-4] Little data are, however, available on the fatty acid (FA) content, composition and accretion rates in fetal adipose tissue throughout gestation. The amount of infant body lipid at delivery shows great inter-individual variation,[1,5-9] which is notably due to a different subcutaneous lipid mass.[10] At term birth of an appropriate for gestational age (AGA) infant, most (~80%) of fetal lipid is located in subcutaneous adipose tissue,[6,11,12] in which lipid by then accounts for 45% of wet weight.[13] An average 3,500 g AGA infant may thus harbor 560 g lipid, of which 448 g is subcutaneous, while the net weight of this compartment amounts to 996 g (i.e. over 25% of total body weight).

Intrauterine fetal fat deposition is best defined by an accelerating quadratic function.[2,14] Typically, adipose tissue development starts with the formation of preadipose cells that resemble empty phospholipid (PL) cell membranes. Long-chain polyunsaturated FA (LC-PUFA, LCP) are preferentially incorporated into PL and therefore mostly found in (adipocyte) cell membranes. Preadipose cells are subsequently filled with lipid in the form of triacylglycerides (TAG) to become mature adipose cells.[15,16] Both saturated (SAFA) and mono-unsaturated FA (MUFA) are predominantly incorporated into TAG. Biopsy samples indicate that preadipose cells are formed up to the 23rd week of gestation, where after their number remains relatively constant, but their size grows continuously.[15-18] Light microscopy studies identified the first traces of adipose tissue lipid deposition around the 15th week after conception.[15] Macroscopically, fat deposition starts from around the 26th week of gestation.[2]

The LCP arachidonic acid (AA) and docosahexaenoic acid (DHA) have received great attention in the last decades because of their abundance in neuronal tissues and their suggested importance in infant neurological development.[19-21] Although LCP reach their highest relative amounts in brain and liver, the much larger adipose tissue and lean skeletal muscle compartments contain the largest absolute amounts.[22,23] Consequently, infant adipose tissue LCP have been considered to be a potential postnatal source of DHA.[24,25] However, little data have been published on the LCP composition of adipose tissue during the last trimester of pregnancy[22] and during early infancy.[26,27] Moreover, the previously reported values of 7.6 g% AA and 1.63 g% DHA in last-trimester fetal adipose tissue TAG+PL[22] contrast markedly with the composition of adipose tissue TAG from infants up to 2 years postpartum (AA: 0.70 and 0.23 g%; DHA: 0.40 and 0.18 g%).[26,27]

Even less data are available on intrauterine adipose tissue FA accretion rates. The currently available LCP accretion rates[22] are likely to have been overestimated, since they derive from the previously noted remarkably high adipose AA and DHA compositions that[22] were not confirmed by others,[26,27] while also the relative decrease of the adipose tissue LCP content[26] with increasing adipose tissue lipid content[13,28,29] was not taken into account in the calculation of these data. To address the gestational age dependent adipose tissue lipid and FA content, FA composition and FA accretion rates, we collected white adipose tissue (WAT) samples from very preterm, preterm and

term infants ranging from 22 to 43 weeks gestation who had not received oral or parenteral feeding, except for intravenous glucose. For the calculation of the FA accretion rates we used the weight of the total WAT mass of infants at 25 and 35 weeks gestation and of term infants with birth weights ranging from 3,500 to 4,500 g from previously published data,[2,8,9] since we had not measured the total WAT mass of the infants in our study. The raw, unsorted data of this paper were previously published in the PhD-thesis of one of us in 1990.[30]

SUBJECTS AND METHODS

Samples

In the period 1986-1987 small samples of WAT were collected from black fetuses and newborns that were born in Curaçao (the former Netherlands Antilles). They were collected as a part of a perinatal mortality study by Wildschut et al.[31] who performed autopsies on 40 fetuses and newborns. The group was composed of one subgroup of 22 stillborn fetuses and infants (12 males and 10 females), and another subgroup of 18 deceased liveborn neonates (11 males and 7 females). Fourteen of the 18 liveborns died within 3 days, the other four within one week, predominantly of asphyxia and respiratory causes. Immediately after dead, the bodies were brought to the hospital mortuary and kept at 4°C. Permission for necropsy was obtained and in most cases autopsy was performed the next morning. Before autopsy all infants were weighed on a calibrated balance. The subcutaneous WAT samples were obtained from the lower abdominal wall. Gestational ages of the infants were estimated by using the physical characteristics according to Farr et al.[32] and by pathological anatomical assessment.[33] Both estimates did not differ more than two weeks and for statistical analyses the average of the two figures was used. None of the infants had received oral or parenteral feeding, except for intravenous glucose. All adipose tissue samples were immediately put into sealable tubes, and kept at -20° C until analysis in the University Medical Center Groningen in the Netherlands. The study was in agreement with the Helsinki declaration of 1975. The study was approved by a local institutional review board[31] and local ethical standards were taken into account.[31]

Fatty acid analysis

Fifty to 100 mg of accurately weighed frozen WAT was homogenized (Potter apparatus) in chloroform and adjusted to 10.0 or 25.0 ml with chloroform. A volume corresponding with 1 mg WAT was transferred into a Sovirel tube, containing a series of odd-chain numbered FA methyl ester (FAME) internal qualification standards (5:0 up to 17:0; 200 µg of each in 200 µl chloroform) and 1 mg butylated hydroxytoluene (BHT) in 100 µl methanol. The final solution was taken to 600 µl by the addition of chloroform. Transmethylation was performed by the addition of 2 ml methanol-hydrochloric acid solution (methanol-6 mol/L HCl (5:1 v/v)) and heating for 4 hours by 90°C. Extraction of FAME and subsequent quantification with capillary GC with flame ionization detection occurred according to previously described methods.[34] Medium-chain saturated FA (MCSAFA, 6:0 up

to 14:0) were quantified with use of 5:0-15:0 as internal quantification standards.[35] Long-chain FA (≥ 16:0) were quantified on the basis of the added 17:0.[34] FA compositions were expressed in mg/g wet WAT and normalized to g/100 g FA (g%). We defined mg/g wet WAT as the WAT FA content, and g% as the WAT FA composition.

Mathematics and Statistics

We divided the total sample of 40 infants into three groups of 15 very preterm infants (gestational ages 22-29 weeks), 11 preterm infants (29-37 weeks) and 17 term infants (37-43 weeks). The total sum exceeds 40 since three samples were included twice, i.e. two (29 weeks old) very preterm and one (37 weeks) term infant were also included in the preterm group to obtain a mean gestational age of 35 weeks. The middle group was excluded from statistical analyses, since it contained duplicates that were also incorporated into the very preterm and the term groups. Group characteristics and anthropometrics are presented in **Table 1**.

Total fetal white adipose tissue fatty acid contents and increment rates

The total contents and accretion rates of FA in fetal WAT (in mg/week) were calculated using the data from all fetuses/infants (n=40) from the 22nd to the 43rd week of gestation. Since we only sampled fetal subcutaneous WAT, we needed an estimation of the total amount of WAT at various ages of gestation. Widdowson et al.[1-4,11,12] reported the total fetal lipid content in AGA fetuses at various gestational ages, which comprises the amount of lipid in all fetal organs, those in WAT and brown adipose tissue (BAT) included. To obtain the total amount of lipids in combined white and brown adipose tissue we subtracted the calculated lipid contents *(Chapter 6.2)* of the most important fetal organs.[36] Subsequently, we calculated the distribution of adipose tissue between WAT and BAT[37,38] to arrive at the total amount of fetal WAT. The outcomes are presented in supplemental Table 1 and the underlying calculations are explained in the legend. Although no such[36] detailed studies are available for large for gestational age (LGA) infants, it is known that about 50% of the additional weight in these infants is lipid, most of which is located in adipose tissue.[8] To facilitate an estimation of the WAT weights in LGA infants, we therefore assumed that 95% of the additional lipid was stored in adipose tissue. Secondly, we estimated (see Results) an adipose tissue FA content of 60% in a 4,000 g and of 65% in a 4,500 g LGA infant.[13,39] The remaining weight, including lipid and lean tissue,

Table 1. Characteristics and anthropometrics of the included infants

	Very preterm	Preterm	Term
Number	15	11	17
Sexe (% male)	53	64	59
Birth weight (g)	685 ± 167	1.934 ± 1.014	3.030 ± 565
Length of gestation (weeks)	24.6 ± 1.8	34.5 ± 2.6	40.2 ± 1.5
Postconceptional age (weeks)	24.9 ± 2.0	34.9 ± 2.4	40.5 ± 1.7

Values are expressed as mean ± SD

was divided equally among the other organs *(Chapter 6.2)*. As explained in detail in the legend of Supplemental Table 1, we estimated that the amount of lipid stored in total WAT in a 25, 35 and 40 weeks old AGA infant amounted to 12, 151 and 479 g, respectively; while in a term 4,000 g and a term 4,500 g LGA infant, the amount of lipid stored in WAT amounted 716 and 953 g, respectively. To verify the outcome of these calculations, we compared our estimates with the available data for infant adipose tissue from the literature.[8-10] From the great similarity we concluded that our calculated amounts of fetal WAT in preterm, term and LGA infants are of sufficient quality to estimate the FA accretion rates from very preterm to preterm and term infants.

Fetal adipose tissue fatty acid content and increment rates

From the adipose tissue FA contents in mg/g WAT at 25, 35 and 40 weeks gestation, we calculated the increment rates of each FA in mg/g WAT/week. From the WAT FA composition (g%), we calculated the total amount of FA in the total WAT compartment. This was calculated by multiplying the calculated total amount of WAT lipid (**supplemental Table 1**) with the mean WAT FA composition (g%) at the corresponding gestational age. Accretion rates were successively calculated by dividing the change in the total amount of a certain FA in total WAT by the number of elapsed weeks. The increment rates in the first two trimesters were calculated by assuming zero fat at conception. Increment rates in the third trimester were calculated from week 25 to 40 weeks. Since lipid accretion occurs in a quadratic manner,[14] we additionally estimated FA accretion rates during the last 5 weeks of gestation, i.e. from 35-40 weeks gestation.

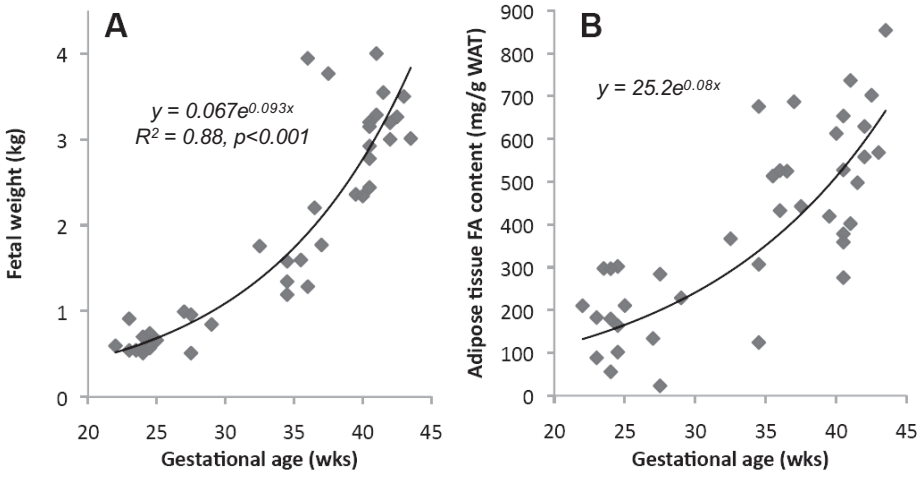

Figure 1. The gestational age dependent changes in fetal weight (A) and the fetal adipose tissue fatty acid (FA) content (B). Data are in kilogram (kg) and mg FA/g wet white adipose tissue (WAT). Fetuses and infants range from 22-43 weeks gestational age.

Statistics

Between-group differences in the FA content (mg/g WAT) and FA composition (g%) of WAT were tested only between very preterm (n=15) and term infants (n=17) with 2-sided independent Student's t-tests and Mann-Whitney U-tests at $p<0.05$. In these two groups there were no duplicate subjects. Finally, we studied for each FA the relations of its content (in mg/g wet WAT) and its relative contribution (composition; in g%) with gestational age and the total WAT lipid content. Relations were explored with regression and curve estimation. Linear, inverse (1/x), logarithmic (log{x}), exponential (x^2) and cubic (x^3) relations were used. All statistical analyses were performed with the aid of SPSS 18.0

RESULTS

Anthropometrics and the relation with adipose tissue fatty acid content

Fetal body weight increased from 685±167 g in the group with an average gestational age of 24.9 weeks to 1,934±1,014 g in the group at 34.9 weeks gestation and subsequently to 3,030±565 g at term birth (40.5 weeks gestation) (Table 1). **Figure 1** shows the relationships between gestational age and fetal weight (A) and between gestational age and the WAT FA content (B). Fetal weight and the WAT FA content as a function of gestational age increased in an accelerating manner, i.e. with highest increment rates in the last weeks of gestation. From 25 to 40 weeks gestation the lipid content of WAT increased from an average of 184 to 547 mg/g AT (**Table 2**). Finally, the adipose tissue FA content increased as fetal weight increased (adipose tissue FA content (in mg/g AT) = 221*ln (fetal weight [in kg]) + 297, R^2=0.56, not shown). For the total fetal WAT mass in LGA infants (see above and supplemental Table 1), we estimated that WAT contained about 600 mg FA/g WAT in a 4,000 g LGA infant and about 650 mg FA/g WAT in a 4,500 g LGA infant.

Fetal white adipose tissue fatty acid content in mg/g AT

The total lipid contents and the FA contents of WAT (mg/g WAT; mean ± SD) at 25, 35 and 40 weeks gestation are presented in Table 2. At 25 weeks, 1 g of WAT contained 83 mg SAFA (16:0>18:0>14:0>10:0), 83 mg MUFA (18:1ω9>16:1ω7>18:1ω7) and 18 mg PUFA (18:2ω6 [linoleic acid, LA]>AA>DHA). Increment rates for SAFA and MUFA were about 4.5 times higher than for PUFA (see also **Supplemental Table 2**). At 40 weeks gestation, 1 g of infant adipose tissue contained 298 mg SAFA (16:0>18:0>14:0>10:0), 226 mg MUFA (18:1ω9>16:1ω7>18:1ω7) and 23.2 mg PUFA (LA>AA>DHA). As can be deducted from Table 2, SAFA (mainly 16:0) and MUFA (mainly 18:1ω9) increased substantially in the 3rd compared to the first two trimesters, while PUFA increased non-significantly between 25 and 40 weeks gestation from 18.0 to 23.2 mg/g WAT; mainly on account of the significantly higher LA (p=0.014) in the 3rd compared to the 1st trimester. Increment rates for SAFA and MUFA during the 3rd trimester were about 30-40 times higher compared to that of PUFA (Supplemental Table 2). LCP increased during the first two trimesters to 7.85 mg/g WAT at 25 weeks gestation, but subsequently remained constant, constituting 7.80 mg/g WAT in a term infant.

Table 2. Adipose tissue fatty acid composition in mg/g wet adipose tissue

	fatty acids (mg/g wet AT)[a]			*regression*[b]	
Fetal age (weeks)	25 (n=15)	35 (n=11)	40 (n=17)	GA	AT-FA
	mean ± SD	mean ± SD	mean ± SD	R^2	
Total FA	184 ± 90.3	439 ± 174	547 ± 155***	0.62[#]	1.00[§]
6:0	0.28 ± 0.37	1.43 ± 0.94	1.97 ± 2.44***	0.21[§]	0.16[§]
8:0	0.45 ± 0.30	0.63 ± 0.25	0.59 ± 0.19		0.34[§]
10:0	4.41 ± 2.17	3.23 ± 0.95	2.78 ± 0.64	0.22[$]	
12:0	0.85 ± 0.36	1.15 ± 0.30	1.29 ± 0.33***	0.25[§]	0.43[§]
14:0	6.50 ± 6.00	15.5 ± 6.62	19.1 ± 4.97***	0.48[‖]	0.62[§]
16:0	61.1 ± 32.1	187 ± 79.9	241 ± 64.4***	0.69[§]	0.97[§]
18:0	9.32 ± 4.56	21.0 ± 8.31	30.4 ± 12.9***	0.57[#]	0.81[§]
20:0	0.04 ± 0.09	0.28 ± 0.21	0.50 ± 0.31***	0.51[§]	0.73[§]
22:0	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.21		
SAFA	82.9 ± 40.9	230 ± 93.5	298 ± 79.9***	0.77[§]	0.98[§]
MCSAFA	12.5 ± 7.10	21.9 ± 7.40	25.7 ± 6.35***	0.44[#]	0.62[§]
LCSAFA	70.4 ± 36.4	208 ± 87.3	273 ± 75.2***	0.77[§]	0.97[§]
14:1ω5	0.42 ± 0.37	1.55 ± 0.82	1.71 ± 0.66***	0.48[§]	0.61[§]
16:1ω7	19.4 ± 11.7	51.5 ± 23.0	57.8 ± 18.3***	0.50[§]	0.81[§]
18:1ω7	5.52 ± 3.21	10.1 ± 3.76	12.0 ± 4.72***	0.35[§]	0.72[§]
ω7	24.9 ± 14.4	61.6 ± 26.6	69.9 ± 22.5***	0.49[§]	0.90[§]
18:1ω9	57.3 ± 28.0	126 ± 51.3	154 ± 52.0***	0.54[#]	0.95[§]
20:1ω9	0.20 ± 0.31	0.48 ± 0.26	0.71 ± 0.37***	0.38[§]	0.51[§]
ω9	57.5 ± 28.1	126 ± 51.5	154 ± 52.3***	0.54[§]	0.96[§]
MUFA	82.8 ± 42.0	190 ± 77.7	226 ± 70.9***	0.61[§]	0.99[§]
18:3ω3	0.09 ± 0.23	0.07 ± 0.23	0.08 ± 0.23		
22:5ω3	0.28 ± 0.70	0.12 ± 0.24	0.11 ± 0.18		
22:6ω3	2.27 ± 1.82	1.87 ± 1.51	1.69 ± 0.93		
LCPω3	2.54 ± 2.11	1.99 ± 1.72	1.80 ± 1.04		
ω3	2.63 ± 2.17	2.06 ± 1.71	1.88 ± 0.97		
18:2ω6	9.89 ± 5.12	11.8 ± 5.48	14.8 ± 6.20*	0.15[¥]	0.51[¥]
18:3ω6	0.21 ± 0.30	0.38 ± 0.30	0.44 ± 0.28*	0.12[§]	0.41[§]
20:2ω6	0.10 ± 0.18	0.24 ± 0.21	0.38 ± 0.24**	0.26[§]	0.48[§]
20:3ω6	0.42 ± 0.50	0.49 ± 0.39	0.67 ± 0.37		0.13[§]
20:4ω6	3.48 ± 2.14	3.56 ± 1.52	3.59 ± 1.52		0.33[¥]
22:4ω6	0.77 ± 0.95	0.57 ± 0.53	0.92 ± 0.46		
22:5ω6	0.53 ± 0.71	0.47 ± 0.39	0.44 ± 0.35		
LCPω6	5.30 ± 4.04	5.34 ± 2.93	6.01 ± 2.75		0.32[¥]
ω6	15.4 ± 8.39	17.5 ± 7.99	21.3 ± 8.92		0.50[¥]
PUFA	18.0 ± 10.1	19.5 ± 9.43	23.2 ± 9.66		0.44[¥]
LCPω3+LCPω6	7.85 ± 6.00	7.33 ± 4.62	7.80 ± 3.66		

Abbreviations: AT, adipose tissue; FA, fatty acid(s); SAFA, saturated FA; MCSAFA, medium chain SAFA (≤14:0); LCSAFA, long-chain SAFA (≥ 16:0); MUFA, monounsaturated FA; PUFA, polyunsaturated FA.

[a], the adipose tissue (AT) fatty acid (FA) content at 40 weeks gestation differs significantly from the composition at 25 weeks gestation at *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$

[b], significant relations ($p<0.05$) obtained after regression analysis for the AT-FA content (mg/g AT) as a function of 1) gestational age (GA), and as a function of 2) the AT-FA content (AT-FA, in mg/g AT). Negative relationships were investigated using [§], linear (y=x) and[‖], inverse (x^{-n}) functions, positive relationships using linear; [¶], logaritmic (ln{x}); [#], exponential (a^{bx}); and [¥], cubic relations (x^3). The indicated value represents the R^2 for the best fitting relation. Relations are shown in Figure 1 and 2.

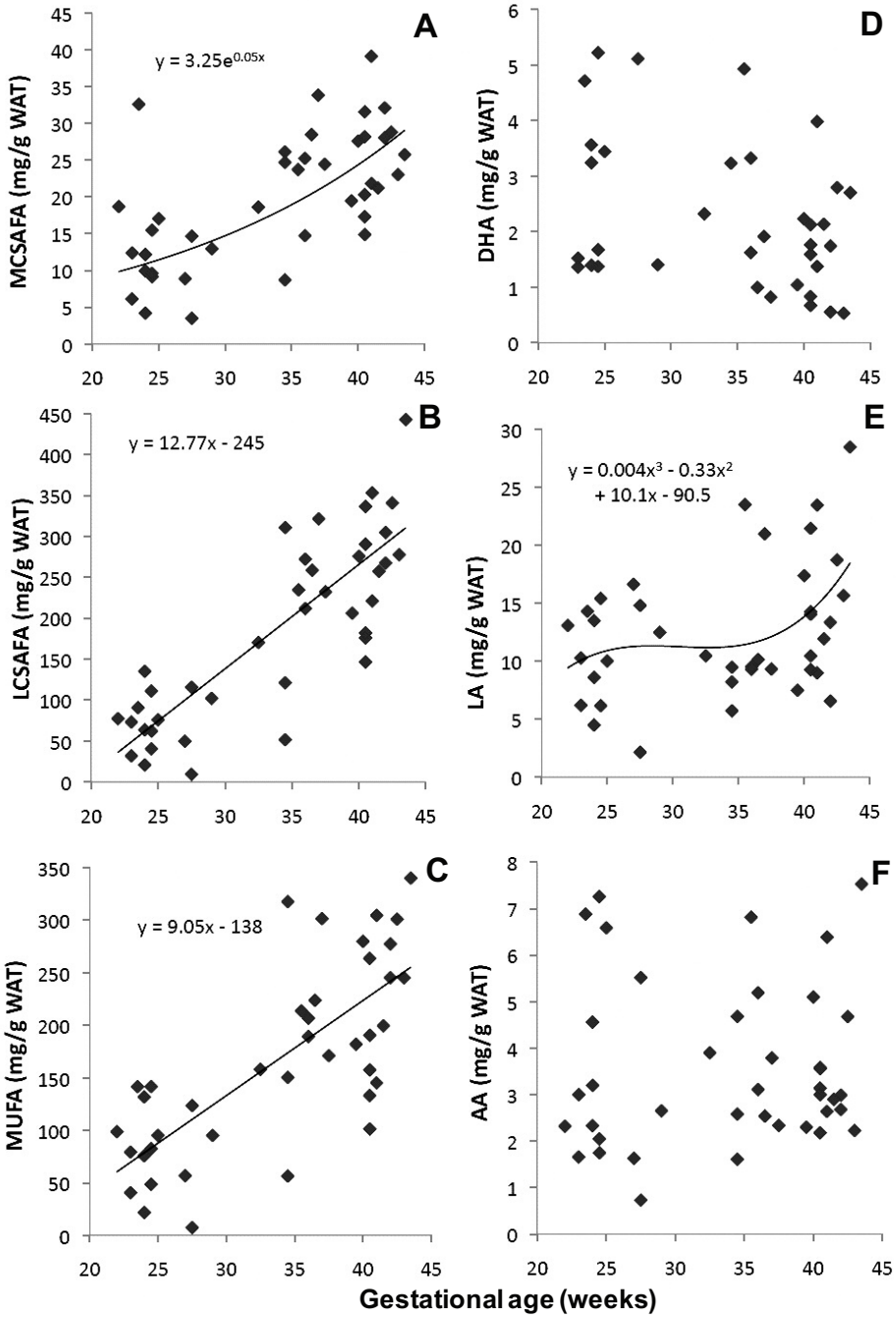

Figure 2. The gestational age dependent changes in the wet white adipose tissue (WAT) medium chain saturated fatty acid (MCSAFA) content (A); long chain saturated fatty acid (LCSAFA) content (B); monounsaturated fatty acid (MUFA) content (C); docosahexaenoic acid (DHA) content (D); linoleic acid (LA) content (E); and arachidonic acid content (AA). Data are in mg FA/g WAT.

While the above describes the between-group differences, we also evaluated these results with regression analyses (Table 2). These revealed comparable results for the relation between the WAT FA content and gestational age (left column under regression in Table 2). **Figure 2A-F** show the changes of some selected FA in mg/g WAT as a function of gestational age. It was found that the WAT MCSAFA contents related exponentially, the long chain saturated (LCSAFA) and MUFA (Figure 2A-C) contents related linearly to gestational age, that those of AA and DHA (Figure 2D and 2F) did not relate to gestational age, while LA exhibited a cubic relation (Figure 2E). To find the strongest contributors to the increase of the WAT FA content, we also related the individual WAT FA to the WAT total FA content (right column under regression in table 2). The relation between the specific FA contents and the sum of FA in WAT was strongest for LCSAFA (notably 16:0) and MUFA (notably 18:1ω9), but insignificant for several PUFA. The ω6-PUFA ($R^2 = 0.50$), notably LA ($R^2 = 0.51$) and AA ($R^2 = 0.33$), and the sum of all PUFA ($R^2 = 0.44$) related positive to the WAT total FA content. However, neither DHA, nor any of the other ω3-PUFA, related to the WAT lipid content.

Fetal white adipose tissue fatty acid composition in g/100 g FA (g%)

Between-group differences (**Table 3**) show the relative increase of SAFA with gestational age (from 45.7 g% at 25 weeks to 54.8 g% at 40 weeks gestation, $p<0.001$), which is explained by an increase in LCSAFA (from 38.21 to 50.04 g%, $p<0.001$), mainly 16:0 (from 32.78 to 44.40 g%, $p<0.001$). MCSAFA decreased (from 7.44 to 4.81 g%, $p<0.001$), mainly on account of 10:0 (from 3.15 to 0.54 g%, $p<0.001$). The relative increase in SAFA also seemed to be at the expense of MUFA (from 43.89 to 40.57 g%, $p<0.009$), mainly 18:1ω9 (from 31.33 to 27.70 g%, $p<0.004$). PUFA also decreased (from 10.27 to 4.26 g%, $p<0.001$), notably on account of DHA (from 1.18 to 0.30 g%, $p<0.004$), 18:2ω6 (from 5.90 to 2.76 g%, $p<0.001$) and AA (from 2.06 to 0.66 g%, $p<0.001$).

The observed between-group differences were also evaluated with regression analysis (Table 3). Subtle differences were observed, with significant decreases for 18:3ω6, 22:4ω6 and 22:5ω6 with regression analysis that were not shown in the between-group statistical analysis. Conversely, the relative increase in 14:0 with gestational age was not confirmed by regression analysis. However, most relations between the adipose tissue FA composition and gestational age became confirmed by regression analysis (Table 3). The relations were even more pronounced when we analyzed the relation between the WAT FA composition and the total WAT FA content. **Figure 3A-F** show the changes in the WAT FA content of some selected FA as a function of the WAT total FA content. Interestingly, a negative relation was observed between MCSAFA and the WAT FA content (Figure 3A), a positive relation with LCSAFA (Figure 3B), but no relation with MUFA (Figure 3C). Most PUFA showed significant (negative) relations with the total WAT FA content (right column under regression Table 3). The inverse relations between DHA, LA and AA and the WAT total FA content are shown in Figure 3D-F.

Table 3: Adipose tissue fatty acid composition in g/100g fatty acids

	fatty acids (g/100 g Fatty acids (g%))[a]			*regression*[b]	
Fetal age (weeks)	25 (n=15)	35 (n=11)	40 (n=17)	GA	AT-FA
	mean ± SD	mean ± SD	mean ± SD	R^2	
6:0	0.12 ± 0.18	0.29 ± 0.15	0.36 ± 0.44*	0.14§	0.14¶
8:0	0.30 ± 0.24	0.14 ± 0.06	0.11 ± 0.02***	0.21§	0.67‖
10:0	3.15 ± 2.22	1.00 ± 0.94	0.54 ± 0.14***	0.65‖	0.79‖
12:0	0.55 ± 0.27	0.29 ± 0.10	0.24 ± 0.05***	0.50‖	0.73‖
14:0	3.32 ± 1.60	3.57 ± 1.01	3.56 ± 0.58*		
16:0	32.8 ± 3.38	41.7 ± 4.20	44.4 ± 2.71***	0.73§	0.51¶
18:0	5.41 ± 1.23	4.89 ± 0.85	5.54 ± 1.25		
20:0	0.01 ± 0.04	0.06 ± 0.04	0.09 ± 0.04***	0.45§	0.42¶
22:0	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.03		
SAFA	45.7 ± 3.83	51.9 ± 3.30	54.8 ± 3.05***	0.65¶	0.30§
MCSAFA	7.44 ± 2.82	5.31 ± 1.31	4.81 ± 0.72***	0.29‖	0.59‖
LCSAFA	38.2 ± 3.71	46.6 ± 3.97	50.0 ± 2.85***	0.75¶	0.43¶
14:1ω5	0.19 ± 0.15	0.34 ± 0.08	0.32 ± 0.09*	0.16¶	0.29¶
16:1ω7	9.50 ± 3.55	11.5 ± 1.40	10.6 ± 1.79		0.31¶
18:1ω7	2.97 ± 0.63	2.35 ± 0.26	2.16 ± 0.44**	0.44‖	0.19§
ω7	12.5 ± 3.71	13.8 ± 1.34	12.8 ± 2.02		0.20¶
18:1ω9	31.3 ± 2.79	29.0 ± 3.31	27.7 ± 3.06**	0.23‖	0.12¶
20:1ω9	0.08 ± 0.12	0.10 ± 0.05	0.13 ± 0.04		0.20¶
ω9	31.4 ± 2.77	29.1 ± 3.27	27.8 ± 3.09**	0.23‖	0.11¶
MUFA	43.9 ± 4.25	43.0 ± 3.11	40.6 ± 3.09**	0.18‖	
18:3ω3	0.04 ± 0.11	0.03 ± 0.10	0.02 ± 0.05		
22:5ω3	0.14 ± 0.39	0.02 ± 0.05	0.02 ± 0.03		
22:6ω3	1.18 ± 0.78	0.39 ± 0.30	0.30 ± 0.12**	0.42‖	0.58‖
LCPω3	1.33 ± 0.96	0.42 ± 0.33	0.32 ± 0.14**	0.38‖	0.16‖
ω3	1.37 ± 1.00	0.45 ± 0.37	0.34 ± 0.12**	0.37‖	0.18‖
18:2ω6	5.90 ± 2.88	2.97 ± 1.34	2.76 ± 1.06***	0.31‖	0.61‖
18:3ω6	0.08 ± 0.11	0.08 ± 0.05	0.08 ± 0.05		0.12‖
20:2ω6	0.04 ± 0.07	0.05 ± 0.04	0.07 ± 0.04		
20:3ω6	0.21 ± 0.24	0.11 ± 0.08	0.12 ± 0.05		
20:4ω6	2.06 ± 0.92	0.88 ± 0.31	0.66 ± 0.17***	0.53‖	0.59‖
22:4ω6	0.39 ± 0.48	0.11 ± 0.10	0.17 ± 0.05	0.15‖	0.13‖
22:5ω6	0.23 ± 0.25	0.10 ± 0.08	0.07 ± 0.05	0.17‖	0.16‖
LCPω6	2.92 ± 1.56	1.24 ± 0.48	1.09 ± 0.30***	0.42‖	0.28‖
ω6	8.90 ± 3.46	4.29 ± 1.65	3.92 ± 1.33***	0.44‖	0.49‖
PUFA	10.3 ± 3.79	4.74 ± 1.88	4.26 ± 1.39***	0.49‖	0.46‖
LCPω3+LCPω6	4.25 ± 2.36	1.66 ± 0.78	1.41 ± 0.38***	0.45‖	0.25‖

For abbreviations: see the legend to Table 2.

[a], the adipose tissue (AT) fatty acid (FA) composition at 40 weeks gestation differs significantly from the composition at 25 weeks gestation at *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$

[b], significant relations ($p<0.05$) obtained after regression analysis for the AT-FA composition (g/100 g FA) as a function of 1) gestational age (GA), and as a function of 2) the AT-FA content (AT-FA, in mg/g AT). For further explanation see also the legend to Table 2

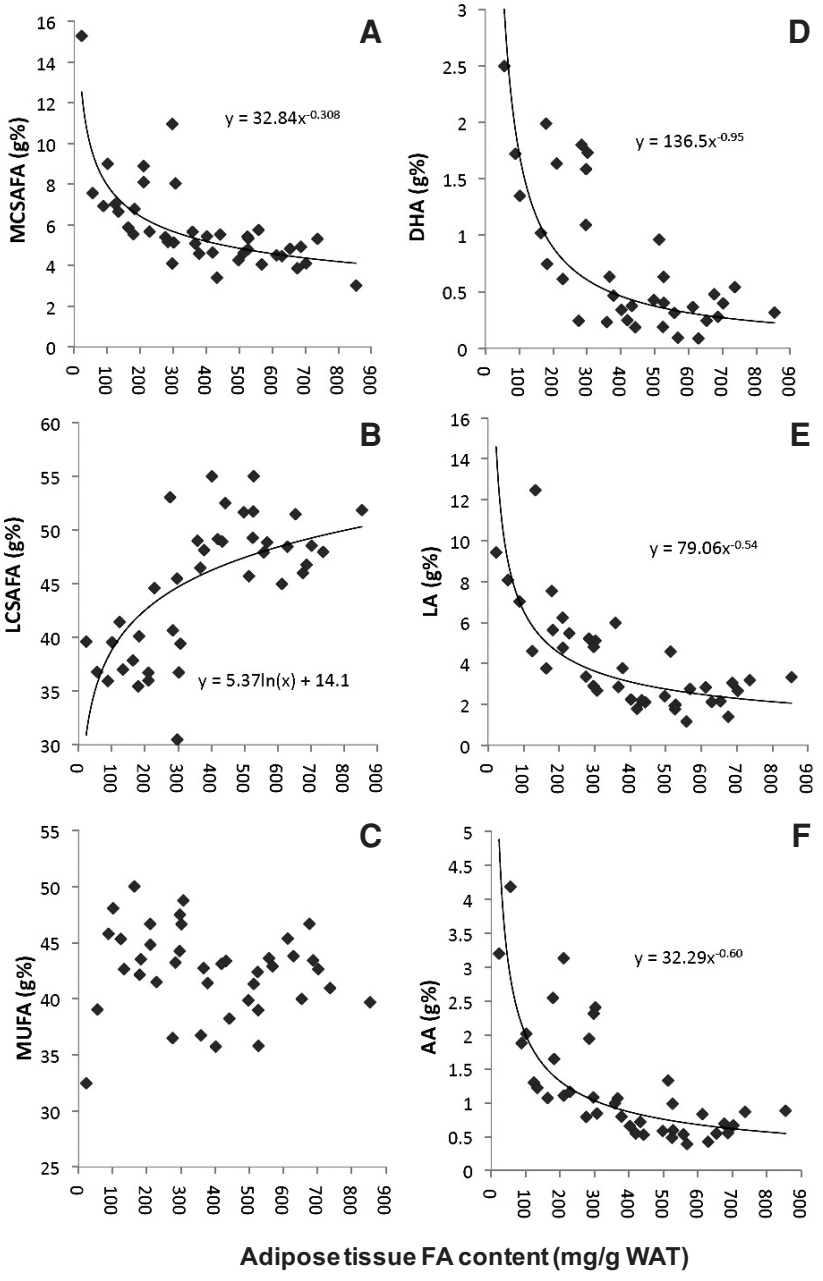

Figure 3. The adipose tissue fatty acid (FA) content (in g/100 g FA, g%) as a function of the wet white adipose tissue (WAT) FA content (in mg FA/g WAT) for MCSAFA (A), LCSAFA (B), MUFA (C), DHA (D), LA (E) and AA (F). For abbreviations see Figure 2.

Total fetal white adipose tissue fatty acid content and fatty acid accretion rates

Using the calculated amounts of total lipid (supplemental Table 1) and the FA composition in g% (Table 3) we calculated the total amount of FA stored in the fetal body at selected gestational age (Supplemental Table 3). Most importantly, there is about 5.52 g SAFA, 5.31 g MUFA and 1.24 g PUFA (including 143 mg DHA and 249 mg AA) in WAT of a 25 weeks old fetus. These amounts increased to 78.3 g SAFA, 64.8 g MUFA and 7.14 g PUFA (including 595 mg DHA and 1,322 mg AA) at 35 weeks and to 262 g SAFA, 194 g MUFA and 20.4 g PUFA (including 1.46 g DHA and 3.15 g AA) in a 3,500 g AGA term infant. We finally calculated that, assuming a similar FA composition and a slight increase in the adipose tissue lipid content (see above), the stored amounts of FA might increase to values as high as 523 g SAFA, 387 g MUFA and 40.6 g PUFA (including 2.90 g DHA and 6.27 g AA) in a 4,500 g LGA infant.

From the total WAT FA content, we calculated that in the first part of pregnancy lipid accreted at 484 mg/week, i.e. 69 mg/day (**Table 4**). This increase in lipids was explained by SAFA (221 mg/week; mainly 16:0), MUFA (212 mg/week; mainly 18:1ω9) and PUFA (49.7 mg/week; mainly 18:2ω6, 28.5 mg/week; AA, 10.0 mg/week; and DHA 5.73 mg/week). During the last trimester, lipid accretion rates increased to 31.1, 46.9 and 62.7 g/week for a 3,500; 4,000 and a 4,500 g term infant, respectively. These increases are mainly explained by SAFA (17.1, 25.8 and 34.5 g/week; mainly 16:0); MUFA (12.6, 19.0 and 25.4 g/week; mainly 18:1ω9) and PUFA (1.28, 1.95 and 2.63 g/week; mainly 18:2ω6, AA and DHA). The accretion rates increased in the last 5 weeks of gestation for SAFA to 36.8; 62.9 and 88.9 g/week; for MUFA to 25.9; 45.2 and 64.4 g/week; and for PUFA to 2.65; 4.68 and 6.70 g/week for infants with birth weights of 3,500, 4,000 and 4,500 g, respectively.

Taken together, our data showed different fetal WAT FA compositions in early, mid and late gestation. All FA showed an accelerating absolute increase, with 2-fold higher increment rates in the last 5 weeks compared to the whole last trimester of gestation. The much higher accretion rate of SAFA (17 g/week) compared to MUFA (13 g/week) and PUFA (1.3 g/week) in e.g. the 3,500 g AGA infant caused a relative dilution of MUFA, PUFA and notably LCP with advancing gestation (Table 3 and Figure 3).

DISCUSSION

We analyzed the FA contents and compositions of fetal WAT of very preterm, preterm and term newborns ranging from 22 to 43 weeks gestation that had not received oral or parenteral feeding, except for intravenous glucose. The amount of lipid/gram of fetal WAT increases substantially during the 3rd trimester, from 18.4 g FA/100 g wet WAT (18.4%) at 25 weeks to 54.7% at term. This increase was on account of SAFA and MUFA, while PUFA, and notably LCP, remained remarkably constant (Table 2). The substantial increase of SAFA caused a relative decrease of PUFA, notably LCP, and to a lesser extent of MUFA (Table 3). Fetal WAT stores contained 1,457 mg DHA and 3,150 mg AA in a 3,500 g AGA term infant, which might increase to 2,901 mg DHA and 6,273 mg AA in a 4,500 g LGA infant (supplemental Table 3). Increment rates for DHA and AA (**Figure 4**) increase from 5.7 mg DHA/

Table 4. Intra-uterine adipose tissue FA increment rates

	FA increment rates (mg/wk and g/wk)[a]								
Period (wk-wk)[b]	0-25	0-25	25-35	25-40	35-40	25-40	35-40	25-40	35-40
ΔFetal BW (g-g)[b]	0-900		900-2525	2525-3500		2525-4000		2525-4500	
Unit	mg/wk	g/wk	g/wk	g/wk	g/wk	g/wk	g/wk	g/wk	g/wk
Total FA	484	0.484	13.89	31.13	65.60	46.93	113.0	62.73	160.4
6:0	0.59	0.001	0.043	0.114	0.256	0.171	0.428	0.228	0.598
8:0	1.47	0.001	0.017	0.033	0.064	0.050	0.116	0.067	0.168
10:0	15.2	0.015	0.113	0.146	0.212	0.231	0.468	0.316	0.722
12:0	2.66	0.003	0.037	0.073	0.144	0.111	0.258	0.149	0.372
14:0	16.1	0.016	0.499	1.107	2.322	1.673	4.022	2.233	5.702
16:0	159	0.159	5.884	13.87	29.84	20.94	51.04	27.94	72.04
18:0	26.2	0.026	0.672	1.723	3.826	2.597	6.446	3.470	9.066
20:0	0.07	0.000	0.009	0.027	0.064	0.041	0.104	0.054	0.144
22:0	0.00	0.000	0.000	0.005	0.014	0.007	0.020	0.009	0.026
SAFA	221	0.221	7.278	17.10	36.74	25.83	62.94	34.50	88.94
MCSAFA	36.0	0.036	0.709	1.473	3.002	2.233	5.282	2.993	7.562
LCSAFA	185	0.185	6.568	15.63	33.74	23.56	57.54	31.49	81.34
14:1ω5	0.90	0.001	0.049	0.100	0.202	0.150	0.352	0.200	0.502
16:1ω7	45.9	0.046	1.615	3.303	6.680	4.983	11.72	6.657	16.74
18:1ω7	14.4	0.014	0.319	0.663	1.350	1.003	2.370	1.349	3.410
ω7	60.3	0.060	1.939	3.966	8.020	5.986	14.08	8.033	20.22
18:1ω9	152	0.152	3.991	8.614	17.86	12.95	30.86	17.35	44.06
20:1ω9	0.41	0.000	0.014	0.039	0.090	0.059	0.150	0.079	0.210
ω9	152	0.152	4.010	8.613	17.82	13.01	31.02	17.41	44.22
MUFA	212	0.212	5.949	12.58	25.84	19.05	45.24	25.45	64.44
18:3ω3	0.21	0.000	0.003	0.005	0.008	0.008	0.018	0.011	0.026
22:5ω3	0.69	0.001	0.001	0.004	0.010	0.007	0.018	0.009	0.024
22:6ω3	5.73	0.006	0.045	0.088	0.174	0.136	0.318	0.184	0.461
LCPω3	6.41	0.006	0.047	0.091	0.180	0.143	0.334	0.193	0.486
ω3	6.62	0.006	0.050	0.097	0.190	0.150	0.350	0.204	0.512
18:2ω6	28.5	0.029	0.377	0.834	1.748	1.273	3.064	1.706	4.364
18:3ω6	0.38	0.000	0.011	0.024	0.050	0.036	0.086	0.048	0.122
20:2ω6	0.20	0.000	0.007	0.022	0.052	0.033	0.084	0.044	0.118
20:3ω6	1.00	0.001	0.014	0.037	0.082	0.055	0.138	0.074	0.194
20:4ω6	10.0	0.010	0.107	0.193	0.366	0.297	0.678	0.402	0.990
22:4ω6	1.89	0.002	0.012	0.050	0.124	0.075	0.202	0.102	0.282
22:5ω6	1.09	0.001	0.012	0.022	0.041	0.033	0.076	0.045	0.112
LCPω6	14.1	0.014	0.152	0.323	0.664	0.495	1.180	0.663	1.686
ω6	43.0	0.043	0.538	1.181	2.468	1.801	4.328	2.421	6.188
PUFA	49.7	0.050	0.590	1.277	2.652	1.951	4.672	2.624	6.692
LCPω3+LCPω6	20.6	0.021	0.199	0.415	0.846	0.639	1.520	0.859	2.180

Abbreviations: wk, week(s); BW, body weight; further abbreviations see Legend to Table 2

[a], calculated from: total body FA at given gestational age/elapsed number of weeks from previous timepoint.

[b], within the given period (wk-wk), fetal BW increased from x to y gram (g-g).

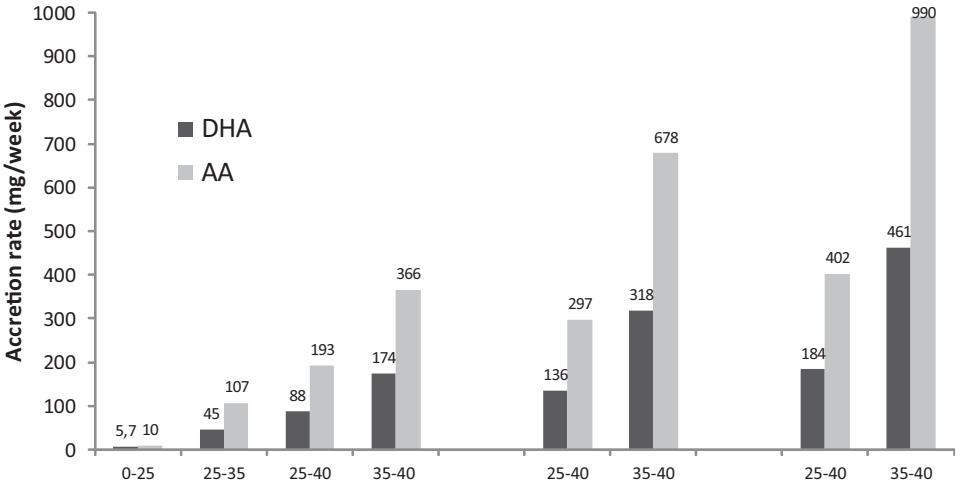

Figure 4. Accretion rates of docosahexaenoic (DHA) and arachidonic acid (AA) in fetal adipose tissue during the first (0-25 weeks) and second part (25-35 weeks, 25-40 weeks and 35-40 weeks gestation) of pregnancy for appropriate for gestational age (AGA) and large for gestational age (LGA) infants. BW, body weight.

week and 10 mg AA/week during the first trimesters, to 88 mg DHA/week and 193 mg AA/week for a 3,500 g AGA infant during the third trimester, with highest accretion rates of 174 mg DHA/week and 366 mg AA/week during the last 5 weeks of gestation. For a 4,500 LGA infant, increment rates are even higher: 184 mg DHA/week and 402 mg AA/week during the 3rd trimester and 461 mg DHA/week and 990 mg AA/week during the last 5 weeks of gestation.

Strengths and limitations

The current data might be used as a reference for the composition of infant WAT at various ages of gestation, although at first sight its extrapolation to the total body WAT FA content might have some limitations. For example, there is some difference between the
fetal body weights at the gestational ages in this study and that of the infants in classical reference works by Widdowson.[1,2,4,11,12] The lower fetal weights at comparable gestational age in our study might be explained by the fact that we studied black fetuses born to women from the Caribbean island of Curaçao. It is known that infants born to black mothers have lower birth weights compared to white women.[40] More importantly however, this lower birth weight has been attributed to lower lean body mass, and not to a difference in adiposity.[41] Since lipid as a percentage of total body weight in Widdowsons classical studies[2,4,11,12] derived from white fetuses, we used the body weight of reference fetuses from Widdowson, instead of the body weights from this study, to calculate the total amount of body lipid, since employment of the body weight of our black fetuses would result in an underestimation of the total amount of body lipid.[41] The estimation of the fetal WAT mass by calculation might be considered as a limitation, but the calculated adipose tissue distributions compared very well with those available in the literature (see legend to supplemental Table 1).

Finally, the current data are to at least some extent influenced by the maternal diet, which in Curaçao might be considered as typically Western, also in 1986-1987.[42] This limitation may notably have affected DHA, of which the status is dependent on maternal fish intake.[43] To which extent the typically high intake of LA in Western countries becomes reflected in current data is unknown, but it is conceivable that maternal LA intakes were comparable with those of Western women.[42] Since none of the infants received oral or parenteral feeding except for intraveneous glucose, the LA status of infant WAT is independent of the known postnatal surge of LA by either human milk or infant formula.[44]

Increase in AT lipid content

The increase in the WAT lipid content during gestation is in line with studies showing the increase in adipose tissue lipid content from 33.5-45.5% in (preterm) newborns[13] to 63-66% for 1-10 months old infants.[39] The exponential relations between the infants' body weight and gestational age (Figure 1A) and its WAT lipid content and gestational age (Figure 1B) are consistent with the fact that the main increases in infant weight and adipose tissue lipid content occurs in late gestation.[2,4,11] Our data also suggest that the heavier term infants have a slightly increased adipose tissue lipid content compared to the lighter ones (figure not shown).

SAFA and MUFA during gestation

After 25 weeks gestation, the increase in the WAT lipid content is on account of SAFA (82.9 to 298 mg/g AT) and MUFA (82.8 to 226 mg/g AT), while PUFA (18.03 to 23.16 mg/g AT), and notably LCP (7.85 and 7.80 mg/g), remain remarkably constant (Table 2, Figure 2). The increase in *de novo* synthesized FA in fetal WAT coincides with increasing insulin secretion from the fetal pancreas after the 26th week of gestation,[11] and with the decreasing maternal insulin sensitivity during late gestation that promotes an increased glucose transport across the placenta.[45] Increasing fetal insulin production and high mother-to-infant glucose transport support fetal *de novo* lipogenesis (i.e. SAFA and MUFA) in liver and adipose tissue.[4] Additionally, FA from maternal *de novo* synthesis are transported across the placenta at increasing rates.[24] Interestingly, the substantial increase of SAFA in fetal WAT (from 45.7 g% to 54.8 g%) caused a relative decrease of PUFA (from 10.27 to 4.26 g%) and notably LCP (4.25 to 1.41 g%), but also of MUFA (from 43.89 to 40.57 g%). The increasing WAT MCSAFA content but their relative decrease suggest that *de novo* lipogenesis aims at LCSAFA rather than MCSAFA, secondary to e.g. insulin induced lipogenic activity in liver and adipose tissue.[46] Since *de novo* lipogenesis is aimed at both SAFA and MUFA, the increase in SAFA but decrease in MUFA suggests that MUFA are selectively transported to e.g. the infant brain for incorporation into neuronal tissues,[21,47] while SAFA, notably LCSAFA, are primarily stored in adipose tissue.

Higher LCP needs in LGA infants

The higher birth weight of LGA infants is mainly caused by increased adipose tissue deposition.[8,9]

Table 5. Comparison of the AT-FA composition and FA accretion rates in the current study to Clandinin et al. (22) and others (26,27)[ab]

	This study						*Clandinin*		*Farquharson*	*Sanjurjo*
	FA (g%) in TL	FA (g%) in TL	FA (g%) in TL	Accretion rates			FA (g%) in TL	Accretion	FA (g%) in TG	FA (g%) in TG
	mean ± SD	mean ± SD	mean ± SD	(mg/wk)			mean ± SEM	rate (mg/wk)	mean ± SD	mean (i.q. range)
Fatty acid	GA 25 wks	GA 35 wks	GA 40 wks	wk 25-35	wk 25-40	wk 35-40	GA 22-40 wks	3rd trimester	GA 30-42 wks	age < 2 years
LA	5.90 ± 2.88	2.97 ± 1.34	2.76 ± 1.06	377	834	1748	3.74 ± 0.63	725	2.20 ± 0.62	13.1 (11.1-13.6)
AA	2.06 ± 0.92	0.88 ± 0.31	0.66 ± 0.17	107	193	366	7.6 ± 1.13	1470	0.70 ± 0.12	0.23 (0.19-0.31)
22:4ω6	0.39 ± 0.48	0.11 ± 0.10	0.17 ± 0.05	12	50	124	1.22 ± 0.42	233	-	0.09 (0.07-0.12)
22:5ω6	0.23 ± 0.25	0.10 ± 0.08	0.07 ± 0.05	12	22	41	0.74 ± 0.35	144	-	0.03 (0.01-0.04)
ALA	0.04 ± 0.11	0.03 ± 0.10	0.02 ± 0.05	3	5	8	0.26 ± 0.15	50.4	0.10 ± 0.01	0.58 (0.47-0.70)
DHA	1.18 ± 0.78	0.39 ± 0.30	0.30 ± 0.12	45	88	172	1.63 ± 0.47	316	0.40 ± 0.04	0.18 (0.10-0.27)

Abbreviations: AT, adipose tissue; FA, fatty acids; TL, total lipid, i.e. the sum of FA in triacylglycerides (TG) and phospholipids (PL); GA, gestational age; wk(s), week(s).

[a], accretion rates in the current and the study by Clandinin et al. (22) apply to AGA infants with a 3,500 g birth weight

[b], note that Clandinin et al. (22) expres the mean ± SEM; while we and Farquharson et al. (26) present mean ± SD; and Sanjurjo et al. (27) the mean with its inter quartile (i.q.) range. Secondly, note the difference in the AT AA content, which is 7.6 g% compared to 0.66-2.06 g% in our data.

LGA infants have a slightly higher adipose tissue lipid content compared to AGA infants, while infants born to mothers with impaired glucose tolerance[8] are likely to harbor even higher amounts of adipose tissue lipids, notably SAFA and MUFA that are synthesized from glucose. However, during adipose tissue development adipocyte hypertrophy is known to plateau, while hyperplasia continues.[29,48] Thus, the increased deposition of SAFA and MUFA in the adipose tissue compartment of LGA infants and infants born to mothers with disturbed glucose tolerance, require a concomitantly increased maternal-to-fetal transport of LCP for adipose tissue hyperplasia to accommodate an additional 250-500 g of lipid. We suggest that the high demands for LCP for incorporation into adipose tissue cell membranes in e.g. LGA infants might compete with the LCP needed for incorporation into the rapidly growing brain, since both adipose tissue and brain rank high in the evolutionary hierarchy, which is also known as the 'survival of the fattest'.[49]

LCP stores and accretion rates compared to the literature

Haggarty[24] suggested that adipose tissue LCP may be considered as a potential postnatal source of DHA, which is conceivable because the quantitatively highest absolute amount of LCP in lipid droplets is located in the TAG fraction.[50,51] Our results suggest that in the AGA infant, about 1,457 mg DHA is stored in WAT, which is somewhat higher compared to the 1,053 as calculated by Cunnane et al.,[23] but substantially lower compared to the 4,166 mg (i.e. 255.6 g FA * 1.63 g% DHA)

reported by Clandinin et al.[22] The discrepancy with the latter data derives from several differences in the outcomes, the most important being the inconsistency with the LCP content of fetal adipose tissue (see also **Table 5**). Clandinin et al.[22] report an average of 1.63 g% DHA and 7.6 g% AA in WAT of infants ranging from 22-40 weeks gestation, which is considerably higher compared to the ranges of 0.30-1.18 g% DHA and 0.66-0 2.06 g% AA that we presently report for infants ranging from 25-40 weeks gestation. The lower DHA and AA contents of fetal WAT in our study is in agreement with an earlier with 1-6 days old preterm and term (30-33 and 37-42 weeks gestation) infants[26] and a study with <2 years old infants.[27] These studies reported fetal adipose tissue DHA compositions of 0.18-0.40 g% and AA compositions of 0.23-0.70 g%, which is comparable to the 0.30 g% DHA and 0.66 g% AA in WAT in the term infants in our study. It should be noted that these two quoted studies reported adipose tissue TAG-FA compositions, whereas in the current we determined the combined (PL+TAG)-FA composition of WAT.

The adipose tissue TAG-FA composition is however similar to that of the adipose tissue (PL+TAG)-FA composition since even in infant AT, with its relatively high contribution of PL, the LCP in PL are unlikely to contribute substantially to the total amount of LCP.[50,51] The discrepancy might relate to the use of packed gas chromatography (GC) columns in the earlier study[22] in contrast to use of capillary GC columns for FA analysis in the latter[26,27] and current study, since use of packed GC columns may produce artificially high LCP values.[52] In addition, the calculations of Clandinin et al.[22] did not take into account the decrease of the adipose tissue relative LCP content[26] with increasing adipose tissue lipid content[13,28] and with gestational age. This decrease was noted both in an earlier[26] and the present study (Figure 3D-F). Clandinin et al.[22] did not specify whether they corrected for these changes, but careful analysis of their data revealed that these were not made*. Finally, the 340 g lipid in a 3,500 g term infant, as estimated by Clandinin et al,[22] is much lower compared to the actual 560 g reported for a term infant by Widdowson.[2] Thus, while the latter might lead to underestimation of FA accretion rates during the 3rd trimester, the above mentioned other 2 discrepancies might explain the higher calculated adipose tissue stores and the higher reported accretion rates.

In conclusion, this study presents the changing fetal WAT FA content and composition with gestation. It provides insight into the physiological development of adipose tissue and gives an estimation of infant FA requirements during gestation. At delivery, WAT from a 3,500 AGA infant contains about 1,457 mg DHA and 3,150 mg AA, while LGA infants might have deposited up to 2,901 mg DHA and 6,273 mg AA in their WAT. We calculated that the average WAT accretion rate for DHA (Figure 4) increases from 5.73 mg DHA/week during the first trimesters, to 88 mg DHA/week for a 3,500 g AGA infant and to 184 mg DHA/week for a 4,500 LGA infant during the 3rd trimester.

* The following example illustrates that no correction for a changing adipose tissue FA composition with increasing gestational age was made. Their[22] Table I shows that infant white adipose tissue increases from 3.45 g in a preterm to 255.6 g at term. Subsequently, the AA accretion rate is calculated by: 255.6 * 7.6 g% (=19.43 g) minus 3.45 * 7.6 g% (=0.26 g) = 19.16 g AA increase during the last trimester = 19.16/13 (where 13 is the weeks in 3rd trimester) = 1.470 mg AA/week; similarly, for DHA the accretion rate is 255.6 * 1.63 g% (=4.17) - 3.45 * 1.63 g% (=0.06) = 4.11/13 = 316 mg DHA/week during the last trimester. These accretion rates are the same as those given in their Table III and results are similar for the other fatty acids.[22]

Accretion rates for AA (Figure 4) increase from 10.0 mg AA/week during the first trimesters, to 193 mg AA/week for a 3,500 g AGA infant and to 402 mg AA/week for a 4,500 LGA infant during the 3rd trimester. Increment rates during the last 5 weeks of gestation were about 2-fold higher compared to the rates during the 3rd trimester. The knowledge on the FA accretion rates in AT, and notably LCP, might be important for the design of nutritional regiments for preterm infants

Supplemental Table 1. Changes in the intra-uterine fetal adipose tissue mass and its lipid content during gestation

Fetal characteristics					
Fetal age (weeks, days)	25 (175)	35 (245)	40 (280)	40 (280)	40 (280)
fetal total body weight (g)	900 [a]	2525 [a]	3500 [a]	4000	4500
% Lipid of fetal body weight (%)	2.3 [a]	7.8 [a]	16 [a]	20.3 [b]	23.6 [b]
Total body lipid (g)	20.6 [a]	198 [a]	560 [a]	810 [c]	1060 [c]
Lipid in internal organs, brown and white adipose tissue (AT)					
Sum of internal organ lipids [excl. internal AT] (g)	7.16 [d]	32.4 [d]	60.4 [d]	63.8 [d]	69.1 [d]
Total adipose tissue lipid (g)	13.4 [e]	166 [e]	500 [e]	738 (746) [f(e)]	975 (991) [f(e)]
Total brown AT lipid (g)	1.47 [g]	15.4 [g]	21.9 [g]	21.9 [g]	21.9 [g]
Total white AT lipid (g)	11.9 [h]	151 [h]	479 [h]	716 [h]	953 [h]
Fetal wet AT weights during gestation					
Adipose tissue lipid (% of wet AT)	18.4 [i]	43.9 [i]	54.7 [i]	60 [j]	65 [j]
Total wet AT weight (g)	73.7 [k]	378 [k]	915 [k]	1230 [l]	1500 [l]
% AT of total body weight	8.2 [m]	15 [m]	26.1 [m]	30.8 [m]	33.3 [m]
Subcutaneous vs. non-subcutaneous AT					
% Lipid as subcutaneous AT (%)	50 [n]	70 [n]	80 [n]		
Total subcutaneous AT lipid (g)	10.3 [o]	139 [o]	448 [o]		
Total deep body (internal) lipid (g)	10.3 [p]	59.4 [p]	112 [p]		
Internal AT lipid (g)	3.26 [q]	27.4 [q]	52.4 [q]		
Total subcutaneous AT mass (g)	56	316	819		
Total internal AT mass (g)	17.7	62.5	95.9		

[a], Adapted from Widdowson[2]
[b], Calculated: total lipid/fetal total body weight (TBW)
[c], Calculated: 50% of the additional weight in a large for gestational age (LGA) infant is lipid[8,9]
[d], Calculated: see Chapter 6.2 for explanation
[e], Calculated: total body lipid - sum of internal, non-adipose tissue, lipid
[f], Calculated: total wet AT weight * AT lipid % (1230*0.60 and 1500*0.65); the difference between the two indicated outcomes is explained by the lack to correct for an increase in the lipid content of organs besides AT in LGA infants
[g], Merklin[37] states that at 25 weeks gestational age (GA), there is 8 g brown AT and at term about 40 g brown AT in the fetal body
[h], Calculated: total AT lipid - brown AT lipid
[i], This study
[j], This study, plotted from the available data (figure not shown, see results for its function)
[k], Calculated: total AT lipid/At lipid %
[l], Calculated: assuming that 95% of the additional lipid in a LGA infant is in AT: hence 0.95*250=238 g lipid + the original 500 g lipid in AT = 738 g, which equals 738/0.60 = 1230 g wet AT. Similar calculation for a 4500 g LGA.
[m], Calculated: total wet AT weight/fetal TBW
[n], Adapted from Southgate & Hey[6], and Widdowson, Southgate & Hey[11]
[o], Calculated: total body lipid * % lipid as subcutaneous AT
[p], Calculated: total lipid - total subcutaneous AT lipid
[q], Calculated: total deep body lipid - internal organ lipid [excl. internal AT]

Legend to supplemental Table 1

Calculation of the total white adipose tissue weight in very preterm, preterm and AGA infants.

Fetal weight ranges from 900 g in a 25 weeks old very preterm infant; to 2525 g in a 35 weeks old preterm infant, while we employed an average birth weight of 3,500 g from an appropriate for gestational age (AGA) term infant. The average total body weight (TBW) lipid compositions were estimated at 2.3 g% at 25 weeks, 7.8 g% at 35 weeks and 16 g% in a 3,500 g AGA term infant.[1-5,11,12] From these data we calculated 20.6 g lipid in the body of a 25 weeks old very preterm infant, 198 g lipid in the body of a 35 weeks old preterm infant and 560 g lipid in the body of a 3,500 g AGA term infant. To estimate the amount of lipid stored in both subcutaneous and internal white (WAT) and brown adipose tissue (BAT) we calculated the amounts of lipid stored within all fetal fatty body organs other than WAT (i.e. brain, liver, skeletal muscle, skeleton, skin, heart, kidney).

Total adipose tissue lipid = total body lipid – total internal organ lipid (Equation A)

The total lipid content of the mentioned organs derived from a detailed study[53] of the available literature on the lipid content of each of these individual organs. The sum of the internal organ lipid weights, amounted to 7.16 g at 25 weeks, 32.4 g at 35 weeks and 60.4 g at term.[53] The resulting remaining weight of lipid in adipose tissue thus ranged from (20.6 minus 7.16) 13.4 g at 25 weeks, to 166 g at 35 weeks and 500 g at term. Merklin[37] reported that a fetus weighing 1 kg has about 8 g of brown AT, which increases to 40 g at term. When assuming that brown and white AT have a comparable lipid content,[38] the 8 g AT amounts to 8*0.18 (adipose tissue contains 18% lipid at 25 weeks, *this study*)= 1.47 g lipid at 25 weeks gestation, 35*0.44 = 15.4 g at 35 weeks and 40*0.55 = 21.9 g lipid at term. Consequently, there is 13.4–1.47=11.9 g WAT lipid in a 900 g fetus at 25 weeks gestation, 166-15.4=151 g WAT lipid in a 35 weeks preterm infant and 500-21.9=479 g WAT lipid in a 3,500 g AGA term infant. These values were employed in supplemental Table 3 and Manuscript Table 4 to calculate total WAT FA accretion rates. From these WAT and BAT lipid weights, we additionally calculated the wet AT (WAT+BAT) weights. After correction for the adipose tissue water content at various ages of gestation, the total wet adipose tissue masses amounted to 73.7 g in a 25 weeks old 900 g fetus, 378 g in a 35 weeks old 2525 g infant and 915 g in a term AGA 3,500 g infant. These outcomes compared well with the reported range of 398 g AT in the average 2,280 g growth retarded infant and 757 g AT in 3,300 g AGA term infant as measured with MRI.[10] It can also be calculated that adipose tissue comprised 26.1% of the fetal TBW for a term AGA infant, which also complies with the 23.4% of TBW as reported by Harrington.[10] From this Table it can also be calculated that at term 89% of lipid is in adipose tissue. Southgate and Hey[6] reported earlier that at 25 weeks gestation body lipid is divided equally between subcutaneous and deep body sites,[2,6,12] but that at term 80% of body lipid is subcutaneous. We therefore used 50%, 70% and 80% subcutaneous adipose tissue at 25, 35 and 40 weeks respectively to arrive at 10.3, 139 and 448 g subcutaneous adipose tissue lipid respectively. These adipose tissue lipid weights are in general agreement with those of Southgate and Hey,[6] who reported that subcutaneous lipid increased from around 20 g at 28 weeks to 350 g at term. When considering that lipid comprises 55% of infant adipose tissue at term (this study), 448 g lipid also complies with the 381 g lipid (from 693*0.55=381) in a 3,300 g term infant.[10] The difference between the total lipid weight and the weight of subcutaneous lipid, was explained by the weight of lipid stored internally. Calculated internal lipid weights ranged from 10.3 at 25 weeks to 112 at term and comply well with the reported amounts of 10 g at 200 days and 80 g at term as described by Southgate and Hey.[6] The analysis of lipid stored in fatty organs other than adipose tissue allowed for the calculation of the amount of internal lipid stored in adipose tissue. We calculated 3.26 g internal adipose tissue lipid at 25 weeks gestation, 27.4 g at 35 weeks gestation and 52.4 g at term. These subcutaneous and internal adipose tissue lipid weights correspond with subcutaneous wet adipose tissue weights of 56 g at 25 weeks gestation; 316 g at 35 weeks gestation and 819 g at term; and with internal wet adipose weights of 17.7 g at 25 weeks, 62.5 g at 35 weeks and 95.9 g at term. The calculated 96 g wet internal adipose tissue at term complies reasonably with the 64 g internal adipose tissue as reported by Harrington.[10]

Calculation of the total amount of white adipose tissue in large for gestational age infants.

To calculate the amounts of adipose tissue in large for gestational age (LGA) infants we used a different approach. Large for gestational age (LGA) infants were set at 4,000 g and 4,500 g at delivery. Hammami[8] and Schmelze[9] showed that the additional weight in a LGA infant is about 50% lipid. Thus when total lipid amounts to 560 g

in an AGA 3,500 g infant it will increase to 810 g (560+{4,000-3,500}/2) and 1,060 g in a 4,000 and 4,500 g LGA infant, respectively. From these amounts of lipid we retrospectively calculated that lipid amounted 20.3 and 23.6 g% of TBW in these infants, respectively. These values compare well with the 905 g and 980 g in 4,244 and 4,430 LGA infants.[8,9] Next, we assumed that 95% of the additional lipid is stored in adipose tissue. Thus, in a 4,000 g LGA infant 500 g of additional weight is 250 g, 95% of which (238 g) is additionally stored in AT. Likewise, in a 4,500 LGA infant 475 g is additionally stored in AT. Since in a 3,500 AGA infant 500 g lipid was stored in AT (Table 2), the sums equal 738 and 975 g AT lipid in a 4,000 and 4,500 g infant, respectively. Secondly, we estimated that in LGA infants, the adipose tissue lipid content increased to 60% in a 4,000 g LGA and to 65% in a 4,500 LGA infant.[13,39] Thus, the amounts of total wet adipose tissue are equivalent to (738/0.60) 1,230 and (975/0.65) 1,501 g AT in a 4,000 and 4,500 g LGA term infant, respectively. When assuming that >90% of lipid is in adipose tissue in a term infant (see above), these amounts compare well with the (905*0.90/0.62) 1,300 g AT in a 4,244 g LGA,[8] the (980*0.9/0.65) 1,350 g AT in a 4,430 g LGA[9] and the 1,720 g AT in a 4,475 g LGA infant born to a mother with impaired glucose tolerance.[9] We redistributed 95% of lipid in fetal adipose tissue, thus 5% remains for redistribution in other fatty organs. Secondly, the 238 g and 475 g adipose tissue lipid equal 396 and 731 g wet adipose tissue, thus there is (500-396) 104 g and (100-731) 269 g weight left, (250*5%) 13 g and (500*5%) 25 g of which is lipid. We redistributed this lipid and remaining lean weight proportionally between the other body organs. However, for the calculation of the amount of lipid in these organs we assumed that the lipid% in these organs remained the same. We calculated that the amount of lipid in these organs amounted to 63.8 g and 69.1 g in a 4,000 g and a 4,500 g LGA infant, respectively. The marginal differences between the directly calculated (738 and 975 g, respectively) total adipose tissue lipid and those amounts calculated with use of Equation A (746 and 991 g, respectively) confirm that the average increases in the organ lipid contents are negligible for our purpose.

Supplemental Table 2. Intra-uterine AT-FA increment rates

	Fatty acid increment rates (mg/g AT/wk)		
Period	trim. 1&2	trim. 3	wk 35-40
Total FA	7.35	24.2	21.7
6:0	0.01	0.11	0.11
8:0	0.02	0.01	-0.01
10:0	0.18	-0.11	-0.09
12:0	0.03	0.03	0.03
14:0	0.26	0.84	0.72
16:0	2.44	12.0	11.0
18:0	0.37	1.41	1.89
20:0	0.00	0.03	0.04
22:0	0.00	0.01	0.02
SAFA	3.32	14.4	13.7
MCSAFA	0.50	0.88	0.76
LCSAFA	2.82	13.5	12.9
16:1ω7	0.78	2.56	1.27
18:1ω7	0.22	0.43	0.39
ω7	1.00	3.00	1.66
18:1ω9	2.29	6.42	5.53
20:1ω9	0.01	0.03	0.05
ω9	2.30	6.45	5.57
MUFA	3.31	9.54	7.26
18:3ω3	0.00	0.00	0.00
22:5ω3	0.01	-0.01	0.00
22:6ω3	0.09	-0.04	-0.04
LCPω3	0.10	-0.05	-0.04
ω3	0.11	-0.05	-0.04
14:1ω5	0.02	0.09	0.03
18:2ω6	0.40	0.33	0.62
18:3ω6	0.01	0.02	0.01
20:2ω6	0.00	0.02	0.03
20:3ω6	0.02	0.02	0.04
20:4ω6	0.14	0.01	0.01
22:4ω6	0.03	0.01	0.07
22:5ω6	0.02	-0.01	-0.01
LCPω6	0.21	0.05	0.13
ω6	0.62	0.39	0.76
PUFA	0.72	0.34	0.73
LCPω3+LCPω6	0.31	0.00	0.10

Abbreviations: AT, adipose tissue; FA, fatty acid; trim., trimester; wk, week; SAFA, saturated FA; MCSAFA, medium-chain FA; LCSAFA, long-chain saturated FA; MUFA, mono-unsaturated FA; LCP, long-chain polyunsaturated FA; PUFA, polyunsaturated FA.

Supplemental Table 3. Intra-uterine AT-FA content

	Fatty acid content (g/infants' total wet WAT)[a]				
	25	35	40	40	40
Fetal BWt	900	2525	3500	4000	4500
Total FA[b]	12.1	151	479	716	953
6:0	0.01	0.44	1.72	2.58	3.43
8:0	0.04	0.21	0.53	0.79	1.05
10:0	0.38	1.51	2.57	3.85	5.12
12:0	0.07	0.44	1.16	1.73	2.30
14:0	0.40	5.39	17.0	25.5	33.9
16:0	3.96	62.8	212	318	423
18:0	0.65	7.37	26.5	39.6	52.7
20:0	0.00	0.09	0.41	0.61	0.81
22:0	0.00	0.00	0.07	0.10	0.13
SAFA	5.52	78.3	262	393	523
MCFA	0.90	7.99	23.0	34.4	45.8
LCFA	4.62	70.3	239	358	477
16:1ω7	1.15	17.3	50.7	75.9	101
18:1ω7	0.36	3.55	10.3	15.4	20.6
ω7	1.51	20.9	61.0	91.3	122
18:1ω9	3.79	43.7	133	198	264
20:1ω9	0.01	0.15	0.60	0.90	1.20
ω9	3.80	43.9	133	199	265
MUFA	5.31	64.8	194	291	387
18:3ω3	0.01	0.04	0.09	0.13	0.17
22:5ω3	0.02	0.03	0.08	0.12	0.15
22:6ω3	0.14	0.59	1.46	2.18	2.90
LCPω3	0.16	0.63	1.53	2.30	3.06
ω3	0.17	0.67	1.62	2.42	3.23
14:1ω5	0.02	0.51	1.52	2.27	3.02
18:2ω6	0.71	4.48	13.2	19.8	26.3
18:3ω6	0.01	0.12	0.37	0.55	0.73
20:2ω6	0.00	0.07	0.33	0.49	0.66
20:3ω6	0.02	0.16	0.57	0.85	1.13
20:4ω6	0.25	1.32	3.15	4.71	6.27
22:4ω6	0.05	0.17	0.79	1.18	1.58
22:5ω6	0.03	0.15	0.35	0.53	0.71
LCPω6	0.35	1.87	5.19	7.77	10.3
ω6	1.08	6.46	18.8	28.1	37.4
PUFA	1.24	7.14	20.4	30.5	40.6
LCPω3+6	0.51	2.50	6.73	10.1	13.4

[a], Total body FA content * lipid FA composition (g%)
b, From Supplemental Table 1

CHAPTER 6.2

Fetal intrauterine whole body linoleic, arachidonic and docosahexaenoic acid contents and accretion rates

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ABSTRACT

Introduction. There is no information on the whole body fatty acid (FA) contents of preterm infants, although scattered information on the FA-composition of many organs is available.

Material and Methods. We collected data on the weights, lipid contents and FA-compositions of the quantitatively most important fetal organs of appropriate for gestational age (AGA) Western infants. From these we estimated the total body contents of linoleic (LA), arachidonic (AA) and docosahexaenoic (DHA) acids at 25, 35 and 40 weeks of gestation

Results. Western infants accrete FA in the order of LA>AA>DHA at all stages during pregnancy and the highest accretion rates are reached in the last 5 weeks of gestation, i.e. 342 mg LA, 95 mg AA and 42 mg DHA/day. At term, most of the infant's LA, AA and DHA is located in adipose tissue (68, 44 and 50%, respectively), with substantial amounts of LA also located in skeletal muscle (17%) and skin (13%); of AA in skeletal muscle (40%) and brain (11%); and of DHA in brain (23%) and skeletal muscle (21%). The term AGA infant has accreted about 21 g LA, 7.5 g AA and 3 g DHA, which constitutes a gap of 12 g LA, 3.3 g AA and 1.5 g DHA compared to a 35 weeks old AGA infant.

Conclusion. The current fetal LA, AA and DHA pool sizes and accretion rates may especially be useful to estimate the preterm infant's requirements and the maternal LCP needs during pregnancy. Since they derive from populations with typically Western diets they do not necessarily reflect 'optimality' or 'health'.

INTRODUCTION

Lipids are important constituents of our body and at term age about 50% of our dry body weight is lipid.[1-3,54] The long-chain polyunsaturated (LCP) fatty acids (FA) are important for infant growth, visual maturity and cognitive development,[55,56] but their importance is not unequivocally demonstrated in randomized controlled trials.[57-59] LCP are constituents of phospholipids (PL) in all cell membranes and of triacylglycerides (TG) in lipoproteins and storage sites. Among the various organs, brain contains the highest *relative* amounts of LCP. However, at any given time, most of the brain is water and although its lipid content increases with age, the about 400 g infant brain at term contains only 9.8 g lipid.[36,60] The absolute and relative docosahexaenoic acid (DHA) contents in brain increase especially in the last trimester of pregnancy. In contrast, the relative amount of brain arachidonic acid (AA) decreases, but it is conceivable that the absolute content increases due to brain growth. Little attention has been paid to the observation that, because of its size, higher quantities of LCP become incorporated into fetal adipose tissue compared to fetal brain,[24] while substantial amounts of LCP, notably AA, are located in the fetal liver.[61] The total amount of LCP accreting in the fetus is currently unknown. This information may be important to estimate the minimum requirements of pregnant women and infants who are born preterm. Pregnancy causes maternal LCP losses, notably DHA, that are associated with postnatal depression,[62] while, dependent of postnatal feeding regimen, premature birth may cause a sizeable LCP "gap" that develops in the period between birth and term age.[63]

The gestational age dependent whole body LCP content can be estimated from the sum of LCP in all LCP-rich organs, such as brain, liver, adipose tissue and (other) lean tissues (e.g. skeletal muscle, skin and the skeleton) at different time points in fetal development. Since after 25 weeks gestation most LCP are stored in adipose tissue, skeletal muscle and brain,[26] it is particularly important to have accurate estimates of the lipid contents and FA compositions of these three organs. The currently available data for such calculations are, however, limited, occasionally inaccurate, or based on gross estimates by the inclusion of animal data. For example, a pivotal study[22] that is frequently used for the calculation of the whole body LCP content[24,25,64,65] suffers from an overestimation of the fetal adipose tissue LCP contents.[54] Finally, there are especially few usable data for calculating the LCP contents of the remaining lean body tissues. The approaches used by Clandinin[22] and Cunnane[23] to estimate the amount of LCP in lean body tissue created a substantial uncertainty level. Clandinin et al.[22] employed a 4% fat content for all remaining wet lean tissue (i.e. all tissues with the exception of brain, cerebellum, spinal cord, liver and adipose tissue) and assumed its FA-composition to be similar to that of human skeletal muscle: i.e. containing 24% ω6-FA and 1.0% ω3-FA.[22] To circumvent the same lack of available data, Cunnane et al.[23] estimated from a 'comparable whole body animal study' that the remaining lean body tissue (i.e. all tissues with the exception of brain, liver and adipose tissue) contained 25% of the liver DHA contents at all times. The thus calculated 44% of whole body DHA in the 'remaining lean tissue' is therefore uncertain.

In the current study we estimated the linoleic acid (LA), AA and DHA contents of a 'reference

fetus' from a population with 'typically Western' dietary habits. Estimates were done for the whole body contents of these FA at 25, 35 and 40 weeks of gestation. We calculated organ weights and their lipid contents and multiplied these with reported LA, AA and DHA contents to arrive at whole organ FA contents that subsequently summed to a whole body FA contents. From these data, we estimated the FA-accretion rates from conception to 25 weeks, from 25 to 35 weeks gestation and during the last 5 weeks of gestation. Gestational ages at 35 and 40 weeks were chosen because FA increment rates in adipose tissue[2] and brain[60] are highest between 35 and 40 weeks.

MATERIALS AND METHODS

All data were calculated for appropriate for gestational age (AGA) infants at the fixed gestational ages of 25, 35 and 40 weeks.

Fetal organ weights

Many of the presently employed data derive from the classical studies of Widdowson and co-workers, who described the chemical and anatomical compositions of fetuses and infants at the various stages of development.[1-5,11,12,36,66,67] First, we estimated total body weights of AGA infants at 25, 35 and 40 weeks gestation.[2,4] Next we collected data on the contributions of the quantitatively most important fetal organs to total body weight, i.e. skeletal muscle, skeleton, skin, liver, and brain[2] and heart, lung and kidneys[68] (**Table 1**, Fetal organ weight (%) of total body weight). From the available data we were able to reconstruct the absolute weights of the quantitatively most important fetal organs during gestation (Table 1, Fetal organ weight). If the reported data did not match exactly with the 25, 35 and 40 weeks gestation of our infants, we inter- or extrapolated linearly from the available data. No data were available for the relative adipose tissue weights during gestation. Consequently, the relative contribution of the adipose tissue mass (Table 1, Fetal organ weight (%) of total body weight) had to be calculated in retrospect from its absolute weight at various ages of gestation. We therefore estimated the absolute weight of the adipose tissue mass at various ages of gestation in AGA infants. A comprehensive explanation of this calculation is given in Supplemental Table 1 of Chapter 6.1). In short, the weight of the wet adipose tissue compartment derives from the difference between the reported[2] total fetal lipid content (Table 1; Fetal organ lipid content (g); total body) and the sum of the lipid weights of the other organs (Table 1, Fetal organ lipid content (g); sum of non-adipose lipid), which was subsequently corrected for the adipose tissue water content at the various gestational ages.[54] To support the outcomes of our calculations on relative and absolute organ weights with the available literature we compared with some detailed *post mortem*[2,22,60,66,68-70] and live born[8-10] studies that provided data for the weights of the organs of preterm and term infants during gestation. The outcomes of our calculations, including those for the weight of the adipose tissue compartment during gestation,[8-10] compared very well with these literature data (see also Chapter 6.1).

Table 1. Fetal organ weights and organ lipid contents

Fetal age (weeks)	25	35	40	
Fetal weight (g)	900	2525	3500	[a]
Fetal organ weight (%) of total body weighy				
Skeletal muscle	25	25	25	[b]
Skeleton	21	19	18	[b]
Skin	13	14	15	[b]
Liver	4	5	5	[b]
Brain	13	13	13	[b]
Heart	0.6	0.5	0.5	[c]
Lungs	2.5	2.0	1.9	[c]
Kidneys	1.5	1.2	1.2	[c]
Adipose tissue	8.2	15	26	[d]
Sum	*88*	*93*	*104*	
Fetal organ weight (g)				
Skeletal muscle	225	631	875	[e]
Skeleton	189	480	630	[e]
Skin	117	354	525	[e]
Liver	32.5	99.5	119	[f]
Brain	104	265	371	[g]
Heart	5.4	12.6	17.5	[e]
Lungs	19.2	46.3	62.1	[e]
Kidneys	13.5	30.3	42.0	[e]
Adipose tissue	73.7	378	915	[d]
Sum	*760*	*2250*	*3494*	
Missing	*15.5*	*10.9*	*0.2*	
Fetal organ lipid content (g/100 g wet weight)				
Total body	2.3	7.8	16	[b]
Skeletal muscle	1.4	1.8	2.0	[h]
Skeleton	0	0.15	0.14	[b]
Skin	1	3	5	[i]
Liver	2.09	3.37	4.01	[j]
Brain	1.40	1.79	2.37	[j,k]
Heart	1.8	1.8	1.8	[k]
Lungs	1.8	1.8	1.8	[k]
Kidneys	1.9	1.9	1.9	[k]
Adipose tissue	18.4	43.9	54.7	[l]
Fetal organ lipid content (g)				
Total body	20.7	197	560	[m]
Skeletal muscle	3.15	11.4	17.5	[m]
Skeleton	0	0.72	0.88	[m]
Skin	1.17	10.6	26.3	[m]
Liver	0.68	3.35	4.77	[m]
Brain	1.46	4.74	8.79	[m]
Heart	0.10	0.23	0.32	[m]
Lungs	0.35	0.83	1.12	[m]
Kidneys	0.26	0.58	0.80	[m]
Adipose tissue	13.6	166	501	[m]
Sum of non-adipose lipid	*7.16*	*32.4*	*60.4*	[m]

[a], from Widdowson[1-2]; [b], from Widdowson[2]; [c], from Phillips[68]; [d], Chapter 6.1 Supplemental Table 1; [e], Calculated from fetal organ weight (%) of total body weight; [f], Mean value of Widdowson, and Coppoletta & Wollbach[2,69]; [g], Mean value of Blinkov & Glezer, and Widdowson & Southgate[60,66];

[h], Extrapolated from White et al.[36], and Dickerson & Widdowson[67]; [i], Extrapolated from Lampe et al. and Williams et al.[71-72]

[j], Calculated from Martinez[73]; [k], from White et al.[36]; [l], from Chapter 6.1

[m], Calculated from fetal organ weight and organ lipid content (g/100 g wet weight)

Fetal organ lipid contents

To estimate the amounts of lipid in organs other than adipose tissue, we collected data on the lipid contents (Table 1, Fetal organ lipid content) of the total body,[2,4] skeletal muscle[36,66] skeleton,[2] skin,[71,72] heart and kidneys.[36] It is in this respect important to note that in the studies that provided data for the FA composition of the skin, the subcutaneous adipose tissue was carefully removed during preparation of the skin samples.[71,72] Again, when the reported data did not match exactly with the 25, 35 and 40 weeks gestation of our reference infants, we inter- or extrapolated linearly from the reported data. If several studies reported data for the same fetal age, we employed their mean. The lipid content of wet adipose tissue at various times of gestation derived from our earlier study.[54] Data regarding the lipid composition of whole wet brain and liver derived from a study reporting nmol FA/g wet tissue in developing infants.[73] We used this study, since it was the only study reporting both lipid contents and the individual organ FA contents of both preterm and term infants. Secondly, we believe that this study by Martinez[73] provides the most accurate data, since a whole homogenized hemisphere was used, while e.g. Farquharson et al.[19] measured the composition of cortex grey matter from the parietal cortex, and Makrides et al.[74] collected samples of frontal lobe, including cortex and underlying white matter. To transform nmol FA/g wet tissue to the actual organ lipid weight we employed an average molecular weight of 292 g/mol for the sum of all FA (total FA).

From the organ weights and the relative lipid contents of the organs we calculated the absolute amount of lipid in each organ (**Table 2**, Fetal organ lipid content). To evaluate these outcomes, we compared with other data from the literature. For this we additionally calculated the amount of lipid in each organ as a percentage of dry weight (i.e. after correcting for organ water content), since the lipid percentages for most organs are documented as percentage lipid of dry organ weight. The respective organ water weights, calculated organ dry weights and organ dry weight lipid percentages are presented in Supplemental Table 1. The calculated lipid contents of brain (1.4 and 2.4 g% of wet weight and 17 and 23% of dry weight at 25 and 40 weeks, respectively) comply well with the available data for brain.[36,75,76] These indicate: 1.0-1.5 g% lipid in brain wet weight at 14-18 weeks and 2.6 g% at 40 weeks gestation;[36,75] and 18 and 25 g% lipid of dry brain weight at 25 and 40 weeks of gestation,[76] respectively. For the liver, the calculated 2.1 and 4.0 g% lipid comply well with the reported[36,61] 2.4 and 4.1-5.0 g% lipid of wet liver weight at 28 and 40 weeks, respectively. Hence, the calculated lipid contents of the organs in the reconstructed reference infants compare well with the available literature.

Fetal organ FA composition, organ FA contents, FA distribution among organs and FA accretion rates

To calculate the LA, AA and DHA contents of all organs (Table 2, mg/organ), we extracted the most appropriate data from the literature (Table 2, FA%). To estimate the FA compositions of skeletal muscle, we employed data from infants <2 years of age, since no separate intrauterine data were

Table 2. Fetal organ lipid content. FA composition. FA content and organ distribution of FA

Fetal age (weeks)	25	35	40	25	35	40	25	35	40	25	35	40
Fetal weight (g)	900	2525	3500	900	2525	3500	900	2525	3500	900	2525	3500
Tissue	Lipid content (g)[a]			LA (g%)[b]			LA (mg/organ)[c]			LA/total LA (%)[d]		
Skeletal muscle	3.15	11.4	17.5	21.4	21.4	21.4[e]	674	2430	3743	41	26	17
Skin	1.17	10.6	26.3	10.6	10.6	10.6[f]	124	1124	2783	7.6	12	13
Liver	0.68	3.35	4.77	5.94	7.26	7.92[g]	40	243	378	2.5	2.6	1.8
Brain	1.45	4.74	8.79	0.22	0.24	0.25[g]	3.2	11	22	0.2	0.1	0.1
Adipose tissue	13.6	166	501	5.90	2.97	2.76[h]	802	4930	13828	48	59	68
	Lipid content (g)[a]			AA (g%)[b]			AA (mg/organ)[c]			AA/total AA (%)[d]		
Skeletal muscle	3.15	11.4	17.5	17.2	17.2	17.2[e]	542	1955	3012	50	46	40
Skin	1.17	10.6	26.3	0.14	0.14	0.14[f]	1.6	15	37	0.2	0.4	0.5
Liver	0.68	3.35	4.77	13.6	10.3	8.63[g]	93	345	412	8.6	8.1	5.4
Brain	1.45	4.74	8.79	10.9	9.79	9.22[g]	158	464	810	15	11	11
Adipose tissue	13.6	166	501	2.06	0.88	0.66[h]	279	1461	3302	26	34	44
	Lipid content (g)[a]			DHA (g%)[b]			DHA (mg/organ)[c]			DHA/total DHA (%)[d]		
Skeletal muscle	3.15	11.4	17.5	3.51	3.51	3.51[e]	111	399	614	28	26	21
Skin	1.17	10.6	26.3	0	0	0[f]	0	0	0	0	0	0
Liver	0.68	3.35	4.77	3.62	3.93	4.08[g]	25	132	195	6.2	8.6	6.5
Brain	1.45	4.74	8.79	7.03	7.52	7.76[g]	102	356	682	26	23	23
Adipose tissue	13.6	166	501	1.18	0.39	0.30[h]	160	647	1503	40	42	50

FA, fatty acids; LA, linoleic acid; AA, arachidonic acid; DHA docosahexaenoic acid
a) Calculated from: fetal organ weight*fetal organ lipid content
b) g/100 g FA
c) FA contents (mg/organ) calculated from: organ lipid content (g)*organ lipid FA composition (g/100 g FA)
d) g FA in organ as percentage of whole body FA (in g)
e) Mean of Sanjurjo et al.[27] and Baur et al.[77-78]
f) From Lampe et al.[71,80] and Elias[79]
g) Calculated from Martinez[73]
h) From Chapter 6.1, assuming that white and brown adipose tissue have similar FA compositions

available.[27,77,78] Similarly, data for the FA composition of whole skin samples derived from a study that described the FA composition in skin of 50 year old males,[79,80] although there is compelling evidence of a changing skin lipid composition during gestation.[72] For reasons explained earlier, we used the FA compositions of brain and liver expressed in nmol/g wet tissue from Martinez.[73] This study is amongst the few[19,74] to report the FA composition as a percentage of total FA in the combined PL-fractions, as opposed to the most of studies reporting FA compositions of the various isolated PL fractions.[47,75,76,81] To calculate the FA compositions of brain and liver, we used molecular weights of 280.5 g/mol for LA, 304.5 g/mol for AA and 328.5 g/mol for DHA. The resulting 7.76 g% DHA and 9.22 g% AA in the brain of a term infant, compared well with the reported postnatal 8.5-9.7 g% DHA and the 10.6-10.9 g% AA as reported by Farquharson et al.[19] and Makrides et al.[74] The outcomes of 4.08 g% DHA and 8.63 g% AA in the infant liver were in reasonable agreement with the 6.18 g% DHA and the 8.84 g% AA as calculated from the equations of Farquharson et al.[61] Due to the

Table 3. Total body FA content and accretion rates for a fetus during gestation[a,b]

Gestational age (weeks)	25	35	40	0-25	25-35	35-40
Fetal weight (g)	900	2525	3500	0-900	900-2525	2525-3500
	Total amount (g/body)[c]			Accretion rate (mg/day)		
LA	1.64	8.74	20.73	9.39	101.35	342.49
AA	1.07	4.24	7.57	6.14	45.22	95.25
DHA	0.4	1.53	2.99	2.27	16.25	41.65

[a], data are in g/body and mg/day

[b], calculated from the difference in the whole body FA content/elapsed time

[c], sum from Table 2

similarity of our term infants with data from others, we assumed that our calculations for preterm infants, which could not be compared with data from the literature, were of sufficient quality to be used for additional calculations for body FA accretion rates.

Data for the FA composition of white adipose tissue at various ages of gestation derived from Kuipers et al.[54] Due to the high similarity of the FA compositions of brown and white adipose tissue,[82] we did not differentiate between these two. No data were available for the FA compositions of heart, lung and kidneys. However their essential-FA (EFA) and LCP contents seem negligible (the cumulative amount of lipid in heart, lungs and kidneys amounts to 3.4%, 0.8% and 0.4% of total lipid at 25, 35 and 40 weeks gestation, respectively), when compared to the sum of the EFA and LCP in the other organs, both because of their relatively low weights and lipid contents (see Table 1).[10,28] Thus, the absence of data for heart, lung and kidneys is unfortunate, but unlikely to have a major influence on the calculated sum of FA in the fetal body, although especially lung has a large epithelium surface area, which might contain considerable amounts of LA and LCP-rich PL.

From the FA compositions of the skin, skeletal muscle, brain, liver and adipose tissue and their total lipid contents, we calculated the total amounts of LA, AA and DHA in each of these organs and combined these to arrive at the corresponding amounts in the entire fetal body at 25, 35 and 40 weeks gestation (**Table 3**, Total amount). Accretion rates (0-25 weeks, 25-35 weeks and 35-40 weeks) were calculated from the differences between the whole body amounts of LA, AA and DHA at different gestational ages, divided by the time difference (Table 3, Accretion rate).

RESULTS

Organ and whole body DHA, LA and AA contents

Table 2 and **Figure 1** show the LA, AA and DHA contents in skeletal muscle, skin, liver, brain and adipose tissue, which are organs that together represent 63, 72 and 84 % (Table 1) of the fetal body weight at 25, 35 and 40 gestational weeks, respectively. Concomitant with fetal growth, the amounts of LA, AA and DHA increased in all organs with increasing gestational age, while the relative contributions (g per 100 g FA; g%) either increased (e.g. brain DHA) or decreased (e.g. brain AA). We estimated (Table 3) that at 25 gestational weeks, the whole fetal body contains about 1.6 g LA, 1.1 g AA and 0.4 g DHA, which subsequently increased to 8.7 g LA, 4.2 g AA and 1.5 g DHA, at

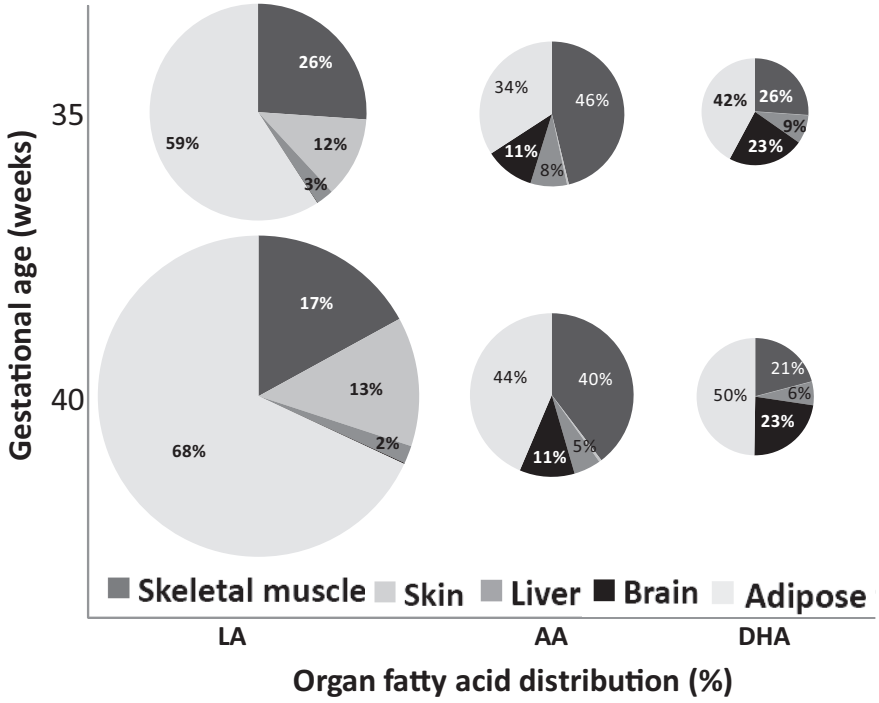

Figure 1. The gestational age dependent linoleic (LA), arachidonic (AA) and docosahexaenoic acid (DHA) content and distribution among the quantitatively most important fetal organs. Organ fatty acid distributions as a percentage (%) of the total body LA, AA and DHA content.

35 weeks, while a 3,500 g term infant harbors about 20.7 g LA, 7.6 g AA and 3.0 g DHA (**Figure 2A**).

LA, AA and DHA organ distribution

Table 2 and Figure 1 show the distributions of LA, AA and DHA among the various organs throughout gestation. Most of the bodily LA was located in adipose tissue and its local abundance increased consistently with advancing gestation (from 48% of the whole body LA pool at 25 weeks to 68% at 40 weeks). This increase occurred mainly at the expense of its location in skeletal muscle (from 41 at 25 weeks to 17% at 40 weeks). With advancing gestation LA in skin increased from 7.6% at 25 weeks to 13% at 40 weeks. Lower percentages of whole body LA were located in the liver, while the LA in brain had a negligible contribution.

Until 35 gestational weeks, most of bodily AA (50% at 25 weeks and 40% at 40 weeks) was located in skeletal muscle (Figure 1). Adipose tissue moved to the preferred location of bodily AA in the last 5 weeks of gestation and at term more AA was located in adipose tissue (44%) than in skeletal muscle (40%). The third most importance location of AA was in brain, but the brain's position of preference decreased with advancing gestation (from 15% at 25 weeks to 11% at 40 weeks). The liver ranked low in the hierarchy of bodily AA and its contribution dropped steeply from 8.1% at 35 weeks to 5.4% at 40 weeks gestation. Skin contributed to whole body AA to a negligible

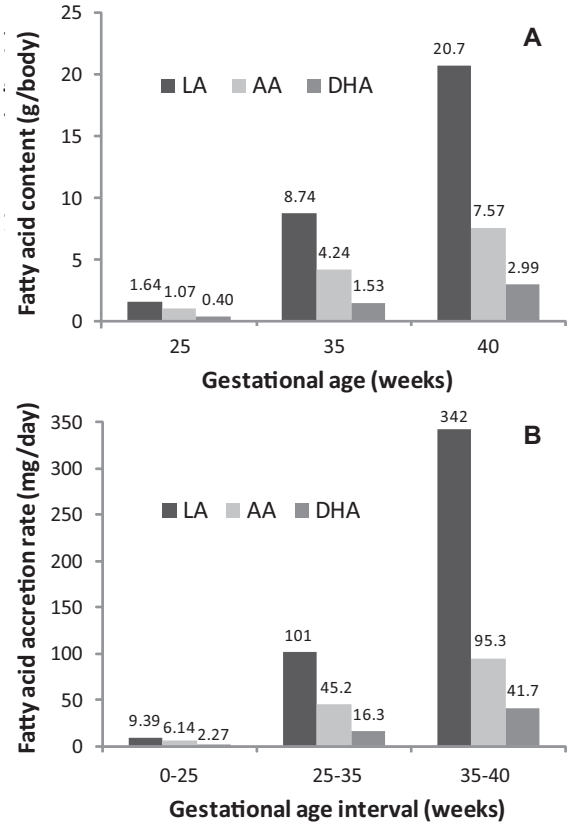

Figure 2. Gestational age dependent **A:** Fetal whole body linoleic (LA), arachidonic (AA) and docosahexaenoic acid (DHA) contents (in g/whole body); and **B:** LA, AA and DHA accretion rates (in mg/day).

extent but at term the contribution had nevertheless increased by 2.5 fold (0.2% at 25 weeks and 0.5% at 40 weeks) (Figure 1).

The majority of bodily DHA was located in adipose tissue (40% at 25 weeks) and its contribution increased with gestation (to 50% at 40 weeks). The second and third most important locations of DHA were in brain (26% at 24 weeks; 23% at 40 weeks) and skeletal muscle (28% at 25 weeks to 21% at 40 weeks). Liver contributed to a seemingly gestational age independent percentage of 6.2-8.6%, while DHA in skin did not contribute to an appreciable extent (Table 2).

LA, AA and DHA whole body amounts and their accretion rates

Table 3 and **Figure 2** show the calculated whole body amounts of LA, AA and DHA and their accretion rates during gestation. The term infant of 3,500 g has accumulated a total amount of about 20.7 g LA, 7.6 g AA and 3.0 g DHA (Figure 2A). LA, AA and DHA accretion rates increased from about 9.4, 6.1 and 2.3 mg/day, respectively, in the first 25 weeks to 101, 45 and 16 mg/day in week 25-35 (Figure 2B). Subsequently, the accretion rates increased 2-3 fold in the last 5 weeks of gestation, to 342 mg LA, 95 mg AA and 42 mg DHA per day (Figure 2B).

DISCUSSION

To estimate fetal whole body LA, AA and DHA contents and accretion rates, we reconstructed 'Western reference infants' at 25, 35 and 40 gestational weeks. At all stages during pregnancy, infants accrete FA in the order of LA>AA>DHA (Figure 2), with the highest accretion rates in the last 5 weeks of gestation (i.e. 342 mg LA, 95 mg AA and 42 mg DHA per day). As expected[1], these accretion rates are lower than earlier estimates of 552 mg ω6-FA and 67 mg ω3-FA per day during the last trimester of intrauterine development.[22] We calculated that a term AGA infant with a body weight of 3,500 g has accreted about 21 g LA, 7.6 g AA and 3 g DHA (Figure 2). The whole body FA 'gap' with an AGA infant at 35 weeks amounts to 12 g LA, 3.3 g AA and 1.5 g DHA. With advancing gestation, LA became increasingly located in adipose tissue (Figure 1). At term whole body LA was distributed in the order adipose tissue>skeletal muscle>skin. Most AA was located in skeletal muscle until 35 weeks of gestation. The contribution of AA in adipose tissue increased rapidly during the last 5 weeks of gestation, and at term AA was located in the order adipose tissue>skeletal muscle>brain (Figure 1). With advancing gestation the contribution of DHA in adipose tissue increased, while that of skeletal muscle decreased. At term, the contribution of DHA in the various organs to whole body DHA declined in the order adipose tissue>brain>skeletal muscle (Figure 1). Our data reemphasize that the widely recognized high relative amounts of DHA and AA in brain do not imply that brain contains the highest absolute amounts of DHA and AA. Adipose tissue seems to be an important storage compartment of LA, AA and DHA.

What current data are and what they are not

Our data derive from populations with typically Western diets and should therefore be viewed upon as 'references' that do not necessarily reflect 'optimality' or 'health'. Human evolution is likely to have occurred in East-African ecosystems where the land meets with water[83,84] and the diet consequently provided considerably lower intakes of LA and much higher intakes of both AA and DHA.[83] We recently showed higher LA in brain and liver (in g per 100 g FA), lower LA in adipose tissue, and both lower AA and higher DHA in brain, liver and adipose tissue in East-African infants who were (still) born to women living close the Victoria Lake (Tanzania) (*Chapter 5*). These women have low intakes of refined vegetable oils and lifetime high intakes of AA and DHA-rich from local fish.[43] Their diets are consequently much closer to the diet on which *homo sapiens'* genome evolved during the 2.5 million years since the rapid growth of our unique brain,[84] suggesting that the LA, AA and DHA contents of their infants are closer to evolutionary optimality than the currently presented.

Adipose tissue as a postnatal mobilizable store

Haggerty[24,25,65] suggested that infant adipose tissue might be a store from which DHA can be mobilized during infant development. It was noted that adipose tissue contains 16-50 times more DHA compared to the infants' brain at delivery.[24,25,65] The present and the earlier study of Cunnane et al.[23] demonstrate that this figure constitutes an overestimation, for which the reasons have been

outlined before,[54] (see also footnote **). In the present study we found that the total amount of DHA in adipose tissue is about 2.2-fold higher than that in brain (Table 2: 1,502 mg/682 mg), which is in reasonable agreement with Cunnane et al.,[23] who calculated a 1.5-times higher DHA in adipose tissue (1,053 mg/720 mg). Nevertheless, the sizeable amounts of DHA in adipose tissue, may be of great benefit to the term infant receiving human milk or a formula with no or low amounts of preformed DHA. Intrauterine accretion of LCP in the fetus is likely to be stimulated by the lower maternal insulin sensitivity with compensatory hyperinsulinemia in the third trimester,[46] the hormones of pregnancy[85] and 'biomagnification'.[86] Each of these influence chain elongation/desaturation via a.o. FADS1 and FADS2 and also transplacental transfer, while their influence becomes instantaneously discontinued after birth. It must, however, be noted that it is currently unknown to what extent the LCP in adipose tissue can become mobilized. Our data, and those of others, refer to the FA in whole adipose tissues and therefore do not distinguish between those located in membrane PL and the intracellular TG droplets. It has also been suggested that LCP may preferentially become mobilized from stores.[87]

Filling the preterm 'gap'

Preterm infants have smaller LCP reserves in adipose tissue (Table 3), which may cause poor ability to compensate for the discontinued maternal supply after preterm delivery. The current brain-centered view may convey the suggestion that, upon LCP shortage, brain ranks highest in the LCP accretion hierarchy. It is, however, questionable whether this is the case. For instance, RBC-LCP are often used as a proxy for the brain-LCP status,[88] which suggests that the available LCP are distributed among many organs. That is, it seems that during LCP shortage the available LCP might in reality be proportionately distributed across a huge distribution volume. Moreover, LCP mobilization from adipose tissue might become inhibited by feeding fortified human milk or special formulas with extra energy and/or protein in the first weeks to months after discharge, a regimen that is often employed to promote catch up growth of preferably lean body mass.[89] During these catch up feeding regimes, a low dietary protein content has been shown to be rate-limiting in the accretion of lean body mass in preterm infants,[89] but it is currently unknown whether an insufficient LCP supply is also rate limiting under these conditions. The suggested inability to mobilize LCP from adipose tissue under such (hypercaloric) catch-up feeding regimes might, especially in the light of the limited postnatal synthesis from precursors,[90] have to become compensated by augmenting the supply of preformed LCP in the diet. This can be achieved by either augmenting the human milk concentration via maternal supplements, or by a high infant formula LCP content. The current LA, AA and DHA pool sizes and accretion rates may be useful to estimate the preterm infant's

** The much higher adipose tissue LCP contents used for this calculation derived from the study that we recently criticized because of: 1) the unusually high LCP contents in adipose tissue of preterm and term infants, 2) the use of packed, as opposed to capillary, gas chromatographic columns, 3) the pooling of preterm and term infants between 22-43 weeks gestation to calculate an average adipose tissue FA content during gestation, while the contribution of LCP to the total FA content in adipose tissue is nowadays known[26,54] to decrease substantially during gestation, secondary to the large influx of the mostly *de novo* synthesized SAFA and MUFA.

requirements, after correction for oxidation and endogenous synthesis and also to estimate the maternal LCP needs during pregnancy.

Linoleic acid, skin and the preterm infant dilemma

Skin seems to become an increasingly important organ for LA deposition with advancing gestation (Table 2). The LA increase occurred in the context of a gestational age dependent growth with concomitantly decreasing surface/volume ratio. Unfortunately, we were unable to locate data for the fetal skin FA-composition. The currently employed skin LA content comes from adult males and is therefore likely to cause an overestimation of the genuine fetal skin LA content. Moreover, the intrauterine environment is characterized by a low LA status since much of the transplacentally transported LA is returned to the mother.[91] LA is an indispensible building block[92] of omega-O-acylceramides, which are unique to the epidermis and responsible for the skin's water barrier function.[92,93] After birth, the ceramide content of the human epidermis increases,[72] while in mice the postnatal LA content of the epidermal ceramides increases 4-fold to reach adult values after 50 days.[94] The increase of LA in skin coincides with the postnatal mother-to-infant LA surge via either the human milk or the infant formula, causing a concomitant rapid increase of the infant's LA status.[44] The low intrauterine LA status may therefore be of importance to preserve maximum communication with the mother via the amniotic fluid while the postnatal LA surge via the milk might be a driving force for the postnatal building of the skin's water barrier.[83] We have previously reported that East-African infants seem to have less trans-epidermal water losses (TEWL) than Caucasian infants immediately after birth, that weight loss on day 1 postpartum was correlated with TEWL, that TEWL was highest in the most premature infants, that TEWL in prematures remained almost constant during the first 4 days after delivery and that large for gestational age infants have higher TEWL than AGA infants of the same gestational age.[95] All of these observations may relate to LA's peculiar behavior in the perinatal phase, with low accretion in the intrauterine period and rapid accretion after birth, when the fetus cannot avoid the sizeable amounts of LA transferred via the milk, as opposed to the fetus-to-mother return of transplacentally transported LA when still residing in the womb.[91]

More data on the genuine LA content of fetal skin will be needed to refine the current model. This is important, since LA might in the above sense be typical for the many dilemma's that surround premature birth, in which one cannot ignore premature exposure to the extrauterine environment but at the same time would like to mimic the intrauterine environment up to corrected term age. The high postnatal LA transfer, especially in Western populations, where a high LA intake is advocated for cardiovascular health,[96] may be of benefit to the rapid limitation of TEWL, but may at the same time compete with both AA and DHA for incorporation into PL and inhibit the conversion of LA and ALA to LCP.[97] A balanced feeding with the minimum amount of LA for the building of a skin water barrier and a sufficiently high AA and DHA supply to compensate for LA's adverse effects seems indicated.

Strengths and weaknesses

The organ weights, as calculated from their percentages of total body mass in one of the classical studies by Widdowson,[1,2] compared well with the outcomes of more recently conducted post-mortem studies.[68,70] Unfortunately, very limited data were available to compare the lipid and FA compositions of various organs during gestation, while for skin and skeletal muscle we were only able to find data on the FA composition after delivery. As outlined above, this is likely to have caused an overestimation of the LA deposits and whole body accretion rate. Nevertheless, the few data that allowed for comparison (see 'Materials and Methods' section) proved highly consistent. Thus, we are confident that the current estimates of the organ weights, lipid contents and FA compositions (those of the adipose tissue compartment included), are of sufficient quality to provide a reasonable estimate of fetal organ FA contents, whole body FA contents and FA accretion rates. Secondly, this is the first report to provide FA accretion rates after reconstruction of an almost complete Western infant, while to our knowledge this is also the first report to include only those data that derive from FA analyses with capillary GC-columns.

The study suffers from some additional weaknesses. The various reports that are at the basis of our calculations employed different analytical methods and included different FA in their sum of total FA. Many of the data are based on limited subject numbers, which may relate to the obvious ethical issues that are inherent to this kind of research. There might have been important dietary differences between populations from which the data have been extracted, while also dietary changes might have occurred in time. Each of these sources of variation in the original data may add up to sizeable differences in the final outcomes. Also, we did not have information of the FA compositions of all organs, while the quantitatively less important organs such as the lungs and the circulatory system have large endothelial surfaces and might thus be expected to be important FA sources that are presently not accounted for. Finally, it is important to reemphasize that we reconstructed an infant from data available from Western infants.

CONCLUSIONS

Western infants accrete FA in the order of LA>AA>DHA at all stages during pregnancy and the highest accretion rates are reached in the last 5 weeks of gestation, i.e. 342 mg LA, 95 mg AA and 42 mg DHA per day. At term, most of the infant's LA, AA and DHA is located in adipose tissue, with substantial amounts of LA also located in skeletal muscle and skin, of AA in skeletal muscle and to a lesser extent in brain, and of DHA in brain and skeletal muscle. The full term AGA infant has accreted about, 21 g LA, 7.5 g AA and 3 g DHA and the 'gap' with an AGA infant of 35 weeks amounts to 12 g LA, 3.3 g AA and 1.5 g DHA. This 'gap' obviously results from the prematurely discontinued transplacental LCP transfer and diminished LCP synthesis from precursors. Limited amount of adipose, hypercaloric postnatal feeding regimens and the low LCP contents in Western human milk and infant formula however, hamper postnatal filling of this 'gap'. The current fetal LA, AA and DHA pool sizes and accretion rates may especially be useful to estimate the preterm infant's requirements

and the maternal LCP needs during pregnancy. Since they derive from populations with typically Western diets they do not necessarily reflect 'optimality' to arrive at 'health'.

Supplemental Table 1. Fetal organ water, dry weight and dry lipid weight

Fetal age (weeks)	25	35	40
Fetal weight (g)	900	2525	3500
Organ water (g/100 g wet tissue)			
Total body	82	73	67 [a]
Skeletal muscle	86.5	82.5	80.4 [b]
Skeleton	69.9	63.8	63.9 [c]
Skin	88.1	84.7	82.8 [c]
Liver	80.7	79.2	78.6 [d]
Brain	91.7	90.4	89.7 [d]
Heart	65.6	84.6	84.1 [c]
Kidneys	88.0	85.2	84.1 [c]
Dry organ weight (g)			
Total body	162	682	1155 [e]
Skeletal muscle	30	110	172 [e]
Skeleton	57	174	227 [e]
Skin	14	54	90 [e]
Liver	6	21	25 [e]
Brain	9	25	38 [e]
Heart	0.8	1.9	2.8 [e]
Kidneys	1.6	4.5	6.7 [e]
Organ lipid content (g/100 g dry weight)			
Total body	12.7	29	48.5 [f]
Skeletal muscle	10.4	10.3	10.2 [f]
Skeleton	0	0.4	0.4 [f]
Skin	8.4	19.6	29.1 [f]
Liver	10.8	16.2	18.7 [f]
Brain	16.8	18.6	23 [f]
Heart	12.9	11.9	11.5 [f]
Kidneys	16.3	12.9	11.9 [f]

[a], adapted from Widdowson[1-2]
[b], adapted from Widdowson & Dickerson[66]
[c], adapted from Widdowson[2]
[d], extrapolated from Widdowson & Dickerson[66]
[e], calculated from organ water (g/100 g wet tissue) and
[f], fetal organ lipid content (Table 1)/dry organ weight

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CHAPTER 7.1

Higher *de novo* synthesized fatty acids and lower ω3- and ω6- long-chain polyunsaturated fatty acids in umbilical vessels of women with preeclampsia and high fish intakes

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ABSTRACT

Umbilical veins (UV) and arteries (UA) of preeclamptic women in Curaçao harbor lower long chain polyunsaturated fatty acids (LCP). The present aim was to test these findings in Mwanza (Tanzania), whose inhabitants have high LCPω3 and LCPω6 intakes from Lake Victoria fish. Women with preeclampsia (n=28) in Mwanza had lower PUFA and higher 20:0 in UV and UA, compared with normotensive/non-proteinuric controls (n=31). Their UV 22:6ω3, 22:4ω6, LCPω6, ω6 and LCPω3+ω6 were lower, while saturated FA, potentially *de novo* synthesized FA (Σde novo) and (Σde novo)/(LCPω3+ω6) ratio were higher. Their UA had higher 16:1ω7, ω7, 18:0 and 16:1ω7/16:0. Umbilical vessels in Mwanza had higher 22:6ω3, LCPω3, ω3 and 16:0, and lower 22:5ω6, 20:2ω6, 18:1ω9 and ω9, compared with those in Curaçao. Preeclampsia in both Mwanza and Curaçao is characterized by lower LCP and higher Σde novo. An explanation might be placental dysfunction, while the similarity of umbilical vessel FA-abnormalities in preeclamptic and diabetic pregnancies suggests insulin resistance as common denominator.

INTRODUCTION

Preeclampsia is a leading cause of fetal and maternal morbidity and mortality in both developing and developed countries.[1,2] Clinically, preeclampsia is characterized by the combination of hypertension and proteinuria. The etiology is as yet poorly understood and there might be multiple pathways involved. Important characteristics are superficial placentation and maternal insulin resistance, dyslipidemia,[3] endothelial dysfunction, and augmented systemic vascular resistance, platelet aggregation and coagulation.[4]

Long chain polyunsaturated fatty acids (LCP; ≥20 carbon atoms with ≥3 double bonds in the *cis* configuration) are either obtained from the diet or synthesized from their parent essential fatty acids (EFA) linoleic (LA; 18:2ω6) and alpha-linolenic (ALA; 18:3ω3) acids by alternating desaturation and elongation. Important LCP are arachidonic (AA, 20:4ω6), eicosapentaenoic (EPA, 20:5ω3) and docosahexaenoic (DHA, 22:6ω3) acids. AA derives from LA, while both EPA and DHA derive from ALA. Generally, AA is abundant in meat, poultry and eggs, while EPA and DHA are abundant in fish, meat and eggs. By their incorporation into phospholipids, LCP are determinants of the physical-chemical properties of membranes and thereby the function of embedded proteins. Their eicosanoid metabolites are involved in signal transduction, while both LCP and their metabolites influence phenotype by interacting with our genome.[5]

We have previously reported that umbilical vessels of preeclamptic women in Curaçao harbor relatively low contents of both LCPω3 and LCPω6.[6] To our knowledge there are no other reports on the fetal LCP status in preeclampsia. A study on the EFA status of mother and child in pregnancy-induced hypertension did not reveal differences in umbilical vessel wall polyunsaturated fatty acids (PUFA), compared with normotensive controls.[7] Recently, the free fatty acid fraction of placental tissue in preeclampsia has been reported to harbor lower LCPω3 (DHA) and LCPω6 (AA),[8] which contrasts with the normal levels of these LCP in the plasma free fatty acid fraction of preeclamptic women as compared to normotensive non-proteinuric women.[9]

The aim of the present study was to test the consistency of our findings in Curaçao. We were interested to see whether studying preeclampsia in a population with high intakes of both LCPω3 and LCPω6 would confirm our previous findings of lower LCPω3 and LCPω6 in preeclamptic vessels. For this we conducted a case-control study on the fatty acid composition of the umbilical vessel walls of preeclamptic and normotensive/non-proteinuretic pregnancies in Mwanza (Tanzania). Mwanza is located on the southern shore of Lake Victoria, which is the world's second largest freshwater lake. The inhabitants of Mwanza are known to have high intakes of DHA, EPA and also AA from the local fish,[10,11] while preeclampsia is a prevalent pregnancy complication in this population.

SUBJECTS AND METHODS

Study design and study groups

Samples were collected by one of us (VJBH) in the labor ward of the Bugando Medical Centre in Mwanza (North-West Tanzania). They were derived from a single study on consecutive preeclampsia

cases within a 6 months period. Samples from apparently healthy pregnant controls were collected in the same period. Matching to preeclampsia cases was based on an as close as possible comparability of gestational age and parity. All investigated women were of Tanzanian descent. Their diets were mainly composed of (mostly cottonseed-oil fried) lake fish and green vegetables, together with "ugali" (maize porridge) or rice. The anthropometric data of the mothers and their children were recorded. Data on gestational age, parity, caesarean delivery and diet were obtained from the medical records, or from translator-assisted interview in Kiswahili by one of us (VJBH). Preeclampsia was defined as a diastolic blood pressure ≥90 mm Hg on ≥2 consecutive occasions ≥4 h apart (or as a diastolic pressure ≥110 mm Hg on any 1 occasion) in previously normotensive women in combination with proteinuria (2 specimens of urine collected ≥4 h apart with ≥0.3 g albumin/L).[12] The final study group was composed of 28 women with preeclampsia, while thirty-one normotensive, nonproteinuric pregnant women served as controls. Their characteristics are presented in **Table 1** for the whole study group. The study was approved by the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/145, Dar es Salaam, dated June 16, 2003 and NIMR/HQ/Vol. IX/369, Dar es Salaam, dated January 17, 2005) and was in agreement with the Helsinki Declaration of 1975 as revised in 2000.

Samples and analyses

Immediately after delivery an approximately 10 cm sample of the umbilical cord, located at the most proximal site to the placenta, was removed and rinsed with a 0.9% NaCl solution. This sample was placed in a cold (4 °C) 0.9% NaCl solution until further processing. The umbilical cord sample was cut into 1 cm pieces. The two arteries and the vein were subsequently dissected from the surrounding tissue, preferably from a 1 cm piece located at 6-8 cm from the placenta. The arteries and vein were thoroughly washed with a cold 0.9 % NaCL solution and transferred to teflon-sealable

Table 1. Clinical characteristics of preeclamptics and normotensive controls

	Controls (n=28)		Preeclampsia (n=31)		*p*
Age (years)	23.7	(15-37)	24.5	(16-37)	ns
Length (m)	1.59	(1.47-1.80)	1.6	(1.47-1.76)	ns
Weight (kg)	58.5	(46.5-88.0)	68.8	(46.0-88.6)	0.001
BMI (kg/m2	23	(19.8-28.1)	26.9	(18.9-31.8)	<0.0001
Upper arm circumference (cm)	24.8	(21.0-30.0)	27.5	(19.5-33.0)	0.002
Diastolic blood pressure (mm Hg)	74	(60-90)	117	(100-170)	<0.0001
Systolic blood pressure (mm Hg)	117	(100-129)	176	(148-220)	<0.0001
Nullipara (%)*	54.8	[17]	57.1	[16]	ns
Caesarian section (%)*	9.7	[3]	60.7	[17]	<0.0001
Gestation age at delivery (weeks)	39	(33.7-41.3)	37.9	(33.9-41.4)	0.03
Infant birth weight (g)	3025	(1600-3800)	2552	(1300-3500)	0.001
Dietary data					
Days of fish/week (number/week)	3	(1-7)	3	(1-7)	ns
Days of meat/week (number/week)	2	(0-7)	2	(1-7)	ns

Data are means (range) unless indicated otherwise. *Numbers in brackets
Between-group comparisons of clinical characteristics were done with Student's t or Chi-square test

tubes that contained 2 mL methanol:HCl (5:1 v/v; 6 mol HCl/L) and 1 mg butylated hydroxytoluene (antioxidant). Fatty acids are stable in this mixture for months.[11]

All samples were stored at room temperature and in the dark until transportation to the University Medical Center Groningen (The Netherlands) for analysis of their long chain fatty acid contents. Analyses of fatty acid methyl esters (FAME) were performed by capillary gas chromatography with split injection and flame-ionization detection, following the addition of an internal quantification standard (17:0 methyl ester), transmethylation and extraction of FAME.[13] Fatty acid compositions were expressed as relative amounts (mol%) and fatty acid ratios in mol/mol. The following fatty acids were determined: SAFAs 14:0, 16:0, 18:0, 20:0, 22:0; ω3-series, 18:3ω3, 20:5ω3, 22:5ω3, 22:6ω3; ω6-series, 18:2ω6, 20:2ω6, 20:3ω6, 20:4ω6, 22:4ω6, 22:5ω6; ω7-series, 16:1ω7, 18:1ω7; ω9-series, 18:1ω9, 20:3ω9. Sums of SAFA, MUFA, PUFA, ω6, ω3, ω3+6, LCPω3, LCPω6, LCPω3+6, Σde novo (i.e. 14:0, 16:0, 18:0, 20:0, ω7 and ω9) were calculated as well as ratios of individual fatty acids.

Statistics

Between-group comparisons of clinical characteristics were tested with Student's *t* test at $p<0.05$.[14] For the detection of between-group differences in fatty acids of the total study population, we first tested whether each of the individual fatty acids or ratios correlated with gestational age in cases and controls, separately, by calculating the Spearman rank coefficient at $p<0.05$. The finding of insignificant correlations in both groups was followed by use of the Mann-Whitney U test at $p<0.05$ for the analysis of between-group differences. The finding of significant correlations in cases, controls, or both, was followed by analysis of covariance (ANCOVA) with gestational age as the covariate at $p<0.05$. We were able to match 21 patients and 21 controls on the basis of gestational age (± 7 days) and parity. For this "matched-group" we tested between-group fatty acid differences with the Wilcoxon Rank test.

The fatty acid compositions of the umbilical arteries and veins of preeclamptic women and their normotensive controls in Tanzania were also compared with those of their counterparts in Curaçao[6] Justification for the comparison with historical data comes from the use of the same fatty acid analysis protocol in our laboratory for more than 25 years and the favorable results of its accuracy and reproducibility.[13] Between-group fatty acid differences for these groups were tested by using the same approach as described for the total study population.

RESULTS

Study population

In both the total study population (Table 1) and the matched-group (data not shown), preeclamptic women had higher postpregnancy weights, body mass indices, upper arm circumferences, maximum diastolic and systolic blood pressures, and percentages caesarian deliveries. In the total study population newborns of preeclamptic women had lower gestational ages and birth weights at delivery. The controls in the matched-group were somewhat younger than the cases (23.5 vs. 24.9

Table 2. Fatty acid compositions in umbilical veins and arteries of normotensiev compared to preeclamptic women

	Total study population									
	Control women (n=31)				Preeclamptic women (n=28)				Significance	
FA	UV		UA		UV		UA		UV	UA
14:0	0.98	(0.64-1.33)	1.09	(0.71-1.48)	0.99	(0.76-1.47)	1.09	(0.55-1.51)		
16:0	26.3	(25.1-31.7)	24.1	(22.3-25.8)	27.0	(24.9-34.3)	23.6	(22.3-29.3)		
18:0	18.59	(17.4-20.1)	19.6	(18.2-21.8)	18.9	(17.1-23.6)	19.9	(18.8-22.0)		0.001*
20:0	0.42	(0.33-0.51)	0.49	(0.40-0.58)	0.46	(0.33-0.54)	0.52	(0.43-0.63)	0.007	0.011
22:0	1.04	(0.89-1.29)	1.45	(1.22-1.61)	1.05	(0.49-1.30)	1.45	(1.30-1.92)		
SAFA	49.6	(47.4-54.8)	49.3	(47.1-50.9)	50.2	(47.7-59.2)	49.2	(47.6-57.9)	0.009*	
16:1ω7	0.73	(0.49-1.30)	0.75	(0.50-1.26)	0.73	(0.53-1.44)	1.04	(0.44-1.61)		0.013
18:1ω7	2.83	(2.12-3.41)	2.78	(2.04-3.85)	2.87	(2.12-3.64)	2.91	(2.03-3.74)		
ω7	3.48	(2.71-4.68)	3.55	(2.77-4.72)	3.55	(2.91-5.08)	3.94	(2.56-4.78)		0.009
18:1ω9	10.1	(7.86-13.9)	11.9	(8.85-16.6)	10.5	(8.70-13.9)	13.3	(8.21-15.9)		
20:3ω9	0.48	(0.23-1.52)	2.58	(0.63-5.40)	0.53	(0.10-1.42)	2.62	(0.30-4.18)		
ω9	14.9	(12.2-21.3)	20.5	(14.5-29.5)	15.5	(12.2-20.2)	21.7	(12.2-26.8)		
MUFA	17.8	(14.4-22.4)	20.0	(15.6-26.7)	18.1	(14.9-22.2)	21.4	(14.2-25.1)		
16:1ω7/16:0	0.03	(0.02-0.05)	0.03	(0.02-0.05)	0.03	(0.02-0.04)	0.04	(0.02-0.07)		0.011
Σde novo	64.0	(61.2-70.1)	67.3	(62.6-76.7)	66.1	(61.8-75.5)	69.4	(64.5-75.9)	0.039*	
Σdn/(LCPω3+6)	2.19	(1.91-2.87)	2.64	(2.07-4.42)	2.41	(2.00-4.04)	2.93	(2.33-4.31)	0.028*	

Table 2 *(continued)*.

	Control women (n=31)				Preeclamptic women (n=28)				Significance	
FA	UV		UA		UV		UA		UV	UA
18:3ω3	0.08	(0.00-0.24)	0.10	(0.00-0.21)	0.06	(0.00-0.17)	0.09	(0.01-0.22)		
22:5ω3	0.32	(0.11-0.95)	0.25	(0.10-0.68)	0.26	(0.01-1.05)	0.20	(0.09-0.64)		
22:6ω3	4.86	(2.96-6.47)	5.22	(4.07-7.51)	4.16	(1.38-7.18)	5.12	(3.02-6.88)	0.036*	
LCPω3	5.15	(3.18-7.42)	5.56	(4.23-8.12)	4.47	(1.40-8.26)	5.30	(3.14-7.48)		
ω3	5.22	(3.28-7.62)	5.60	(4.33-8.26)	4.51	(1.41-8.35)	5.34	(3.26-7.49)		
18:2ω6	2.58	(1.74-3.43)	1.72	(0.95-2.44)	2.61	(1.95-4.30)	1.74	(1.16-2.68)		
18:3ω6	0.00	(0.00-0.05)	0.00	(0.00-0.05)	0.00	(0.00-0.04)	0.00	(0.00-0.01)		
20:2ω6	0.28	(0.19-0.47)	0.18	(0.11-0.26)	0.34	(0.20-0.51)	0.20	(0.11-0.41)		
20:3ω6	1.62	(1.19-2.31)	1.34	(0.84-2.09)	1.58	(1.01-2.02)	1.37	(0.92-1.72)		
20:4ω6	15.3	(12.4-17.5)	12.5	(8.01-15.9)	14.2	(12.1-17.1)	11.4	(8.99-15.0)		
22:4ω6	4.68	(2.85-6.43)	2.46	(1.37-4.35)	4.36	(1.91-5.40)	2.32	(1.58-3.82)	0.029	
22:5ω6	1.83	(1.14-2.85)	2.27	(1.53-4.23)	1.66	(0.75-2.93)	2.39	(1.27-3.69)		
LCPω6	23.9	(19.6-26.3)	19.6	(13.1-24.7)	22.2	(17.1-25.9)	18.1	(13.1-21.6)	0.009	
ω6	26.9	(22.0-28.5)	21.2	(14.4-26.1)	24.7	(19.4-28.7)	19.8	(14.6-23.7)	0.035	
PUFA	33.0	(27.3-35.0)	31.3	(26.2-34.2)	31.0	(22.1-34.2)	28.8	(22.3-32.2)	0.021*	0.020*
LCPω3+ω6	29.3	(24.4-32.1)	25.4	(17.4-30.6)	27.2	(18.6-30.9)	23.6	(17.6-27.7)	0.014*	

Data are in mol%, median (range); FA, fatty acid; UV, umbilical vein; UA, umbilical artery. Statistics: significance of the differences between cases and controls were tested by Mann-Whitney U test or ANCOVA (*) with gestational age as covariate at $p<0.05$. SAFA, saturated FA; MUFA, monosaturated FA; PUFA, polyunsaturated FA; ωx, total of fatty acids of ωx-series; LCPω3+ω6, sum of long chain polyunsaturated fatty acids (≥20 carbon atoms with ≥3 double bonds in the cis-configuration) of the ω3 and ω6 series; de novo FA, potentially de novo FA; Σde novo (sum of 14:0, 16:0, 18:0, 20:0, ω7 and ω9); Σdn/LCPω3+ω6, Σde novo to the sum of LCPω3 and LCPω6.

Table 3. Synoptic overview of the differences in umbilical vein and artery fatty acid compositions between preeclamptic and normotensive controls in Tanzania and Curaçao[a]

	Tanzania		Curaçao	
De novo fatty acids	**UV**	**UA**	**UV**	**UA**
16:0			↑	
18:0	(↑)	↑		
20:0	↑	↑		
SAFA	↑			
16:1ω7		↑	nd	nd
ω7		↑	nd	nd
20:3ω9				↑
ω9				↑
16:1ω7/16:0		↑		
Σde novo	↑			↑
Σdn/(LCPω3+ω6)	↑			↑
PUFA				
22:5ω3			↓	↓
22:6ω3	↓			↓
ω3			↓	↓
LCPω3			↓	↓
20:3ω6			↓	↓
20:4ω6				↓
22:4ω6	↓			↓
ω6	↓		↓	↓
LCPω6	↓		↓	↓
PUFA	↓	↓	↓	↓
LCPω3+ω6	↓		↓	↓

[a], data for Curaçao derive from ref.[6]

↑ / ↓, higher/lower in preeclamptics compared to controls For statistics and abbreviations see legend to Table 2.

years). There were no case-control differences in the frequencies of fish and meat consumption.

Fatty acid compositions of umbilical venous and arterial vessels

Total study population (**Table 2***)*. In the total study population 20:0 was higher, and PUFA lower, in both umbilical veins (UV) and arteries (UA) of preeclamptic women, when compared with controls. In addition, UV of preeclamptic women had lower: 22:6ω3, 22:4ω6, LCPω6, ω6 and LCPω3+ω6, while saturated fatty acids (SAFA), the sum of potentially *de novo* synthesized fatty acids (Σde novo; i.e. sum of 14:0, 16:0, 18:0, 20:0, ω7 and ω9) and the Σde novo/(LCPω3+ω6) ratio were higher. In UA of preeclamptic women 18:0, 16:1ω7, ω7 and 16:1ω7/16:0 were higher. Data on selected LCPω3, LCPω6 and potentially de novo synthesized fatty acids in UV and UA of women with preeclampsia and controls are depicted in **Table 3**.

Matched-group (data not shown). In the matched group 18:0 was higher in both UA and UV of preeclamptic women, compared with controls. In UV of preeclamptic women 20:0 was higher, while 16:1ω7 and 16:1ω7/16:0 were higher in UA.

Comparison of umbilical vessel fatty acids in Tanzania and Curaçao (data not shown)

As compared to their healthy or preeclamptic counterparts in Curaçao, Tanzanian women had higher 16:0 in control UV ($p<0.0001$), control UA ($p=0.003$) and preeclamptic UV ($p=0.009$); higher 22:6ω3, LCPω3 and ω3 in control UV ($p=0.001$; 0.003; 0.004), control UA ($p=0.009$; 0.016; 0.022) and in preeclamptic UA ($p<0.0001$; 0.001; 0.001); lower 22:5ω6 (a marker of DHA insufficiency) in control UV, control UA, preeclamptic UV and preeclamptic UA (all $p<0.0001$); lower 20:2ω6 (marker of high LA-intake) in control UV, control UA (both $p<0.001$) and preeclamptic UV $p=0.006$); lower 18:1ω9 and ω9 in control UV ($p<0.0001$; 0.010), preeclamptic UV ($p=0.044$; 0.027) and preeclamptic UA ($p=0.008$; 0.007). Of additional interest is that levels of 22:5ω6 in UA and UV in controls in Curaçao are significantly higher (bothl $p<0.0001$) compared with Tanzanian preeclamptic UA and UV (data not shown).

DISCUSSION

In the present case-control study, we studied the fatty acid compositions of the umbilical veins (UV) and arteries (UA) of 28 preeclamptic women and 31 healthy pregnant controls living in the fish-eating town of Mwanza (Tanzania).

The aim of the study was to test whether our previous findings of lower LCP status in children born to preeclamptic women in Curaçao (Netherlands Antilles)[6] could be confirmed in a population with relatively high intakes of freshwater fish. The Lake Victoria fishes are known to be rich in both LCPω3 (DHA and EPA) and LCPω6 (AA).[10,11] High LCPω3 intakes from fish by both normotensive and preeclamptic women in Tanzania became indeed confirmed by the data of the dietary questionnaire (Table 1) and the higher DHA, LCPω3 and ω3, and the lower 20:2ω6 (a marker of high LA-intake) and 22:5ω6 (a marker of DHA insufficiency) in both the UA and UV of their offspring, as compared to their counterparts in Curaçao. Despite higher umbilical LCPω3 in Tanzania we found similar preeclampsia-control differences in UA and UV fatty acid compositions as previously reported in women living in Curaçao,[6] although differences in Curaçao were more pronounced (Table 3). Taken together, the data show that umbilical vessels in preeclampsia, in both Tanzania and Curaçao, contain lower PUFA (especially LCP), and higher SAFA and potentially *de novo* synthesized fatty acids than corresponding vessels of healthy control pregnancies.

Preeclampsia is regarded as a heterogeneous disorder, in which the combination of placental dysfunction and maternal metabolic state contribute to the phenotypic expression of the disease. The observed lower LCP content in preeclamptic umbilical vessels fits well with inappropriate placental functioning as less LCP seem to cross the placenta. Indeed, lower placental LCPω3 (DHA) and LCPω6 (AA) contents in preeclampsia have been reported by Wang et al.,[8,9] which was explained by increased placental oxidative stress. It is of interest that the observed pattern of higher potentially *de novo* synthesized fatty acids and lower LCP in preeclamptic umbilical vessels has previously been noted by us in the offspring of women with type 1 diabetes mellitus (T1DM) and gestational diabetes mellitus (GDM) when compared with appropriate controls.[15] It is well known that from the third trimester, the fetus synthesizes large amounts of fat from non-lipid precursors, notably glucose, which accumulate in adipose tissue as 16:0, 18:1ω9 and 16:1ω7.[5,16-19] From our observations[15] we concluded that children born to mothers with poor glucose homeostasis have higher intrauterine *de novo* fatty acid synthetic activity, which tends to 'dilute' the available LCP. Although preeclampsia and maternal diabetes have an insulin resistant state in common, poor glycemic control is not as evident and a feature of preeclampsia *per se*. In other words, the magnitude of the intrauterine *de novo* fatty acid synthesis is likely to be higher in gestational diabetes, compared with preeclampsia. As we did not anticipate this finding, our study protocol did not include fasting glucose and insulin measurements. However, the strong resemblance of diabetic and preeclamptic umbilical fatty acid patterns is in line with the various reports that link preeclampsia to a state of insulin resistance. Insulin resistance, as determined in various ways, has been documented to occur prior to preeclampsia,[20-27] during preeclampsia,[28-32] and months[28,33,34] and years[35-38] after

preeclamptic pregnancy, suggesting that insulin resistance is part of the maternal metabolic state. The association between preeclampsia and insulin resistance persisted in studies that controlled for maternal weight, either by including lean women only,[21] using BMI-matched controls,[22,23,28-32,34,39] or adjustment by multivariate analysis.[20,24,26,27,36-38] A maternal insulin resistant phenotype may perturb maternal fatty acid metabolism and, consequently, fatty acid supply to the fetus. First, it may perturb normal adaptive changes in insulin sensitivity during human pregnancy. That is, it may attenuate the normally higher insulin sensitivity of early pregnancy, which promotes storage of (dietary) fatty acids (e.g. LCP) in maternal adipose tissue.[3,40] Second, it may exacerbate the normal state of insulin resistance in the second half of pregnancy, which drives free fatty acid (e.g. LCP) release from maternal adipose stores, their processing in the liver, and their presentation to the placenta, either as free fatty acids or as part of VLDL-TG.[3,4] Since increased insulin resistance may be accompanied by increased hepatic *de novo* fatty synthesis,[41,42] it is conceivable that a relatively higher proportion of the potentially *de novo* fatty acids in VLDL are presented to the fetus. Donnelly et al.[41] estimated that 30% of the fatty acids in hepatic-triglycerides and VLDL-triglycerides are derived from *de novo* synthesis in non-alcoholic fatty liver disease (NAFLD), which condition is strongly associated with insulin resistance.[43] Although we did not measure insulin resistance in the present study, the similarity between the fatty acid composition of preeclamptic and diabetic umbilical vessels inspires us to speculate that, next to altered placental handling and transport capacity, we are dealing with a common denominator of maternal insulin resistance, causing secondary effects on fetal LCP status with as yet unknown consequences.

CONCLUSIONS

Preeclampsia in Mwanza is characterized by higher contents of potentially *de novo* synthesized fatty acids and lower contents of LCPω3 and LCPω6 in umbilical vessel walls. In essence, we confirmed the previously reported differences between the fatty acid compositions of preeclamptic and healthy pregnancies in Curaçao.[6] We speculate that these differences are derived from a combination of perturbed placental function and insulin resistance. Detailed studies on the underlying mechanisms and their consequences are needed.

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CHAPTER 7.2

Postpartum changes in maternal and infant erythrocyte fatty acids are likely to be driven by restoring insulin sensitivity and DHA status

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ABSTRACT

Introduction. Perinatal changes in maternal glucose and lipid fluxes and *de novo* lipogenesis (DNL) are driven by hormones and nutrients. Docosahexaenoic acid (DHA) reduces, whereas insulin augments, nuclear abundance of sterol-regulatory-element-binding-protein-1 (SREBP-1), which promotes DNL, stearoyl-CoA-desaturase (SCD/Δ9-desaturase), fatty acid-(FA)-elongation (Elovl) and FA-desaturation (FADS). Decreasing maternal insulin sensitivity with advancing gestation and compensatory hyperinsulinemia cause augmented postprandial glucose levels, adipose tissue lipolysis and hepatic glucose- and VLDL-production. Hepatic VLDL is composed of dietary, body store and DNL derived FA. Insulin resistance increases the contribution of FA from hepatic-DNL to VLDL-triacylglycerols; e.g. saturated-FA and monounsaturated-FA (MUFA) in maternal serum lipids increase during pregnancy. Changes in maternal serum and RBC essential-FA (EFA) after delivery are described, but no authors went into detail about non-EFA and the mechanisms behind and functions of observed changes.

Hypothesis. Postpartum FA-changes result from changing enzymatic activities that are influenced by the changing hormonal milieu after delivery and DHA-status.

Empirical data. We studied FA-profiles and FA-ratios (as indices for enzymatic activities) of maternal and infant RBC at delivery and after 3 months exclusive breastfeeding in 3 populations with increasing freshwater-fish intakes. DNL-, SCD- and FADS2-activities decreased after delivery. Elongation-6 (Elovl-6)- and FADS1-activities increased. The most pronounced postpartum changes for mothers were increases in 18:0, linoleic (LA), arachidonic acid (AA) and decreases in 16:0, 18:1ω9 and DHA; and for infants increases in 18:1ω9, 22:5ω3, LA and decreases in 16:0 and AA. Changes were in line with the literature.

Discussion. Postpartum increases in 18:0, and decreases in 16:0 and 18:1ω9, might derive from reduced insulin-promoted DNL-activity, with more reduced SCD- than Elovl-activity that leaves more 16:0 to be converted to 18:0 (Elovl-activity) than to MUFA (SCD-activity). Postpartum changes for ΣDNL, saturated-FA and MUFA related negatively to RBC-DHA. This concurs with suppression of both SCD- and Elovl-6 activities by DHA, through its influence on SREBP. Infant MUFA and LA increased at expense of their mothers. Sustained transport might be important for myelination (MUFA) and skin development (LA). Maternal postpartum decreases in FADS2-, and apparent increases in FADS1-activity, together with increases of LA, AA, and 22:5ω3, but decrease of DHA, confirm that FADS2 is rate limiting in EFA-desaturation. Maternal LA and AA increases might result of rerouting from transplacental transfer to the incorporation into milk lipids and discontinued placental AA-utilization.

Implications. The magnitude of insulin sensitivity and DHA-status may concertedly aim at mother-to-infant transfer of certain FA during gestation and lactation.

INTRODUCTION

Long chain polyunsaturated fatty acids (LCP-FA) of the ω3 (LCPω3) and ω6 series (LCPω6) are important across the entire life cycle,[44] but notably during infant development. LCP can be derived from (fatty) fish (docosahexaenoic acid; DHA, 22:6ω3) and from meat and eggs (arachidonic acid, AA, 20:4ω6) or derive from endogenous synthesis from the parent essential FA (EFA; linoleic acid, LA, 18:2ω6 and alpha-linolenic acid, ALA, 18:3ω3) by consecutive steps of desaturation and elongation, in which FADS1 (Δ5-desaturase) and especially FADS2 (Δ6-desaturase) are rate limiting.[45-48] LCP are abundant in brain[10] and the absolute content of infant brain AA and DHA increase rapidly from the last trimester of pregnancy up to 2 years postpartum.[49] Insufficient LCPω3 intakes have been implicated in suboptimal (neuro)development, cardiovascular disease and (neuro)psychiatric disease.[44] Obstetric complications such as pregnancy-induced hypertension, gestational diabetes, preeclampsia and type 2 diabetes mellitus in pregnancy are characterized by a relative LCP deficiency, that is likely to be caused by the dilution of LCP by fatty acids that derive from glucose by hepatic *de novo* lipogenesis (DNL).[15,50]

Hypothesis

We hypothesize that the changing hormonal milieu at the end of pregnancy and the maternal LCPω3 status might influence the magnitudes of both DNL and LCP-synthesis.

Our attention towards the possible influence of the changing hormonal milieu at the end of pregnancy and the possible influence of LCPω3 on DNL and LCP-synthesis was drawn after we studied the postpartum changes in the RBC non-essential FA and EFA composition of mothers, and their exclusively breastfed infants, with increasing LCPω3 status,[51] as derived from their intakes of tropical freshwater fish[52] and as confirmed by their increasing LCPω3 status.[53] At the time we did not measure fasting insulin and glucose levels, nor did we perform oral glucose tolerance tests to verify insulin sensitivity in the pregnancy-end or after 3 months lactation. However, the decreasing insulin sensitivity with advancing gestation[54] and its rapid restoration thereafter are well known.[55] Secondly, after obtaining our results we believe that our data showed several arguments for the influence of both the hormonal milieu at the end of pregnancy and the influence of LCPω3 on DNL and LCP synthesis. In this article we review the literature and discuss the outcomes of our study in the light of the changing insulin sensitivity during pregnancy and with regard to the influence of the concurrent LCPω3 status.

Evaluation of the hypothesis

Glucose transport across the placenta is unrestricted, while FA cross with more difficulty.[56] There is sufficient evidence to state that maternal insulin sensitivity decreases with advancing gestation.[54,56-59] The decreasing maternal insulin sensitivity and compensatory hyperinsulinemia facilitate transplacental nutrient transfer by causing a unique combination of augmented glucose production,[54] elevated postprandial glucose levels,[54] adipose tissue lipolysis[59] and increased hepatic

VLDL production[59] that is likely to result in part from augmented DNL. Interestingly, one study in an East African population described reduced postprandial glucose levels in pregnant compared to non-pregnant women,[60] but unfortunately did not investigate concomitant insulin levels to evaluate actual insulin sensitivity.

The liver synthesizes VLDL from FA that are derived from the diet, stores and DNL (which is directed at ≥16:0 the liver).[59,61] Within the liver, saturated-FA (SAFA) may become desaturated by stearoyl-CoA desaturase (SCD) to form monounsaturated-FA (MUFA) such as 16:1ω7 and 18:1ω9,[62] while elongation of 16:0, 16:1ω7 and 18:1ω9 by 'elongation of very long chain fatty acids family member 6' (Elovl-6) will form 18:0, 18:1ω7 and 20:1ω9, respectively.[63] High carbohydrate intakes and insulin resistance in humans increase the contribution of FA from hepatic DNL in VLDL-triacylglycerols (TG).[41,42] Animal studies confirm that insulin positively influences the activities of the enzymes involved in DNL (i.e. FA synthase, FAS and acetyl-Coenzyme A carboxylase, ACC), SCD, Elovl-6, and the desaturase enzymes FADS1 and FADS2;[48,61,63,64] while glucose also has positive effects on DNL, SCD and Elovl-6,[61] but negative effects on FADS1 and FADS2 activities.[48] In contrast, DHA is a potent suppressor of DNL, SCD, Elovl-6. and also of FADS1 and FADS2.[45,64] Changes in enzymatic activities have been correlated to their enzyme product/EFA ratios (DNL activity index) and enzyme product/substrate ratios (SCD, Elovl-6, FADS1 and FADS-2 activity indices) in serum lipids[63,65-69] and adipose tissue,[70] although no data are available for these indices in erythrocytes (RBC).

The increasing insulin resistance towards the pregnancy-end causes the 'hyperlipidemia of pregnancy' that is characterized by consistent increases in maternal plasma phospholipids (PL) and RBC SAFA and MUFA.[71-74] These derive from DNL and adipose tissue lipolysis and cause a relative dilution of PUFA with advancing gestation.[71-74] Lower PUFA might in addition be explained by the selective transfer of LCP across the placenta ('biomagnification'), which is in contrast to SAFA, MUFA and their parent EFA.[75]

After delivery, the rapidly changing hormonal milieu and notably the restoring insulin sensitivity,[54,55] may be expected to drive tremendous FA changes that become reflected in the RBC-FA composition. These changes are mostly opposite to those observed during pregnancy. In addition, the nutrient supply becomes rerouted from transplacental transfer to breastfeeding and fat becomes the infant's main energy source. In contrast to the liver, conversion of SAFA to MUFA via SCD or chain-elongation via Elovl-6[76,77] is limited in the lactating breast, and so is FADS-activity.[78] Humans have little capacity to convert EFA to LCP.[79] Consequently, the developing infant derives its LCP mainly from breast milk that on its turn derives the LCP mainly from maternal long term storage organs (70%) and to a lesser extent from the diet (30%).[78,80]

The FA composition of the maternal stores might become reflected in the course of the maternal plasma PL-FA and RBC-FA. Lactating women show postpartum increases of LA, AA, eicosapentaenoic acid (EPA, 20:5ω3) and 22:5ω3 to, and even beyond, pre-pregnancy values, while DHA decreases below preconceptional values.[81] The infant' demands might be reflected in the courses of the infant plasma PL-FA and RBC-FA that show consistent increases of LA, 18:0 and 18:1ω9, and a decrease

of 16:0 after delivery.[82-84] Infants also show a consistent decrease of AA, while DHA showed an apparently milk DHA-concentration-dependent decrease or no change.[84-88]

Empirical data

Study design

We studied[51] the courses of the RBC-FA and their ratios as proxies for enzymatic activities in 187 mother-infant pairs from delivery to 3 months exclusive lactation in 3 ethnic Tanzanian groups with different intakes of LCPω3 from local freshwater fish, i.e. the Maasai (no or very low fish intake), subjects from the Pare Mountains (intermediate fish intake, 2-3 times/wk) and subjects from Sengerema near Lake Victoria (high fish intake, 3-4 times/wk). We collected samples at delivery and at 3 months PP for measurement of RBC-FA. All groups were considered homogeneous with respect to ethnicity/tribe and their lifetime dietary habits. Our earlier[52] and current[89] study on the milk FA composition of these tribes provided us with some information on their dietary habits, since human milk is known[78,80] to reflect long time storage organs (70%) and the maternal diet (30%). Milk[52] and RBC data[51] show that 16:0 is higher in milk from lactating and RBC from non-pregnant/non-lactating Maasai (27.90 and 25.85 g%, respectively) and lower in counterpart Sengerema (19.65 and 23.99 g%, respectively), while Maasai have low milk DHA (0.20 g%) and Sengerema high milk DHA (0.64 g%).

RESULTS

Results for SAFA and MUFA and enzyme activity ratio indices are presented in **Supplemental Tables 1a, 1b, 2a and 2b**. A synoptic overview of the observed differences for the RBC-FA and the enzyme activity indices between delivery and 3 months PP for both mothers and infants is presented in **Figure 1.** All women showed a decrease in the sum of the *de novo* synthesized FA (SAFA + ω7-FA + ω9-FA; ΣDNL-adult) from delivery to 3 months PP. Mothers at 3 months PP had, compared to delivery, lower RBC-16:0, 16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 22:3ω9, 20:1ω9, 22:1ω9, 24:1ω9, 22:5ω6, and DHA. These lower values at 3 months PP were compensated for by higher 18:0, 20:0, 22:0, LA, 18:3ω6, AA, ALA, 20:5ω3 and 22:5ω3. The quantitatively most important positive changes occurred in RBC-LA, AA, and 18:0, the negative changes in DHA, 18:1ω9 and 16:0 (**Figure 2**). *Taken together*, ΣDNL-adult decreased consistently after delivery, mainly on account of 16:0 and MUFA. Importantly, despite their high intake of 16:0 via cow milk, Maasai mothers had the lowest RBC-16:0 at delivery and at 3 months PP. Also, the lower the maternal DHA status at delivery, the more the RBC-DHA status decreased during subsequent lactation, while 22:5ω3 increased most. LA and AA increased in all mothers, independent of DHA status. The indices for DNL, SCD, FADS1, FADS2 and Elovl-6 activities **(**Figure 1**)** at 3 months PP compared to delivery indicated a lower maternal DNL, Δ9- and FADS2 activity but a higher FADS1 and Elovl-6 activity.

In their infants, there was a corresponding decrease in ΣDNL-infant (i.e. ΣDNL-adult minus 14:0 and 18:1ω9, since these derive mainly from maternal breast milk). At 3 months PP, the infants had lower RBC-16:0, 24:0, 18:1ω7, 20:3ω9, 22:3ω9, 20:3ω6, AA, 22:4ω6 and 22:5ω6. Higher RBC-FA at 3

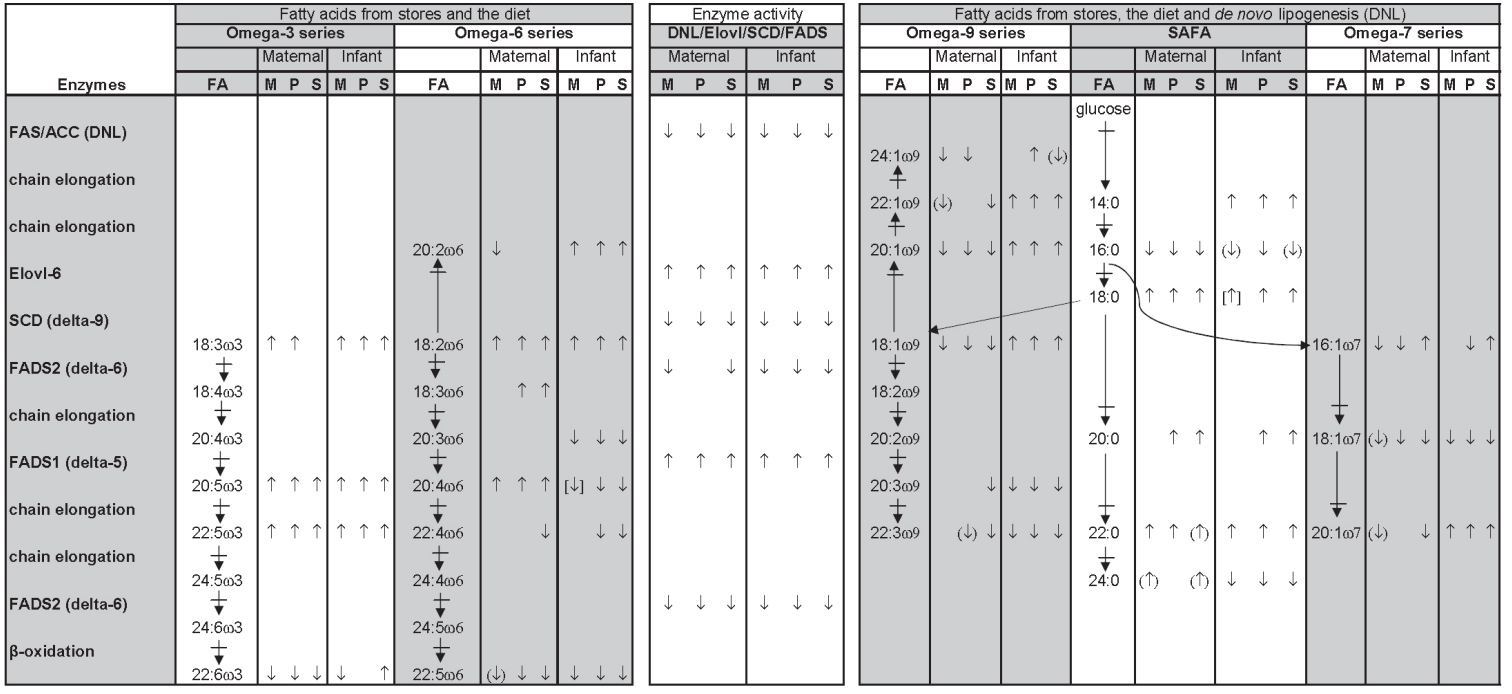

Figure 1. Differences between RBC fatty acids and indices of enzyme activity at delivery and 3 months postpartum for Maasai, Pare and Sengerema mothers and their infants. Abbreviations: M, Maasai; P, Pare; S, Sengerema; ↑ and ↓, higher and lower at 3 months postpartum compared to delivery at $p<0.05$, respectively; (↑), and (↓) higher and lower at 3 months postpartum compared to delivery at $0.05<p<0.10$, respectively; [↑] and [↓], difference higher than 1 g% but insignificant due to low subject numbers.

months PP were noted for RBC-14:0, 18:0, 20:0, 22:0, 20:1ω7, 18:1ω9, 20:1ω9, 22:1ω9, LA, 20:2ω6, ALA, EPA and 22:5ω3. The quantitatively most important positive changes occurred in RBC-LA, 18:1ω9 and 14:0 (Figure 2). The major negative changes were noted for 16:0, 24:0, AA, 18:1ω7 and 20:3ω9. Interestingly, RBC-DHA decreased in those two groups with no and low fish intakes, but increased in the group with high fish intakes.[53] The indices for enzymatic activity indicated a lower infant DNL and FADS2 activity, but a higher FADS1 and Elovl-6 activity at 3 months PP compared to delivery.

Finally, we found that maternal RBC-DHA was inversely related to maternal RBC-ΣDNL(adult) at delivery (**Figure 3A**) and after 3 months lactation (**Figure 3B**). Most individual FA included in the sum of DNL-FA showed similar results (**Supplemental Figure 1**). Generally, a higher maternal DHA status related to higher RBC-16:0, but lower 18:0, 16:1ω7 and 18:1ω9, while infants with the highest DHA status had lower RBC-ΣDNL, mainly on account of lower 16:1ω7 and 18:1ω9 at delivery and 18:1ω9 at 3 months PP.

DISCUSSION & CONSEQUENCES OF THE HYPOTHESIS

The many changes in the RBC-FA from delivery to 3 months PP (Figure 1) are in part consistent with a return to the prepregnancy state[90-92] such as e.g. suggested by the presently observed decreases of 16:0 and 18:1ω9, and the increases of 18:0, LA, AA and EPA. Mechanistically, they might be explained by: 1) the hormonal changes associated with the transition from pregnancy to lactation, 2) the (consequently) changing bidirectional mother-infant transplacental nutrient transport to a unidirectional nutrient transport from mother to infant via the milk, 3) differences in LCPω3, notably DHA, status.

Saturated-, ω7- and ω9-fatty acids

The most remarkable changes in maternal RBC-SAFA, ω7-FA and ω9-FA from delivery to 3 months PP included highly consistent decreases of 16:0, 18:1ω7, 18:1ω9 and 20:1ω9 (and to a lesser extent 16:1ω7, 20:1ω7, 22:1ω9, 24:1ω9, 20:3ω9 and 22:3ω9) and on the other hand, the increases in 18:0, 20:0, 22:0, and to a lesser extent 24:0 (b**).** These changes indicated no change of maternal SAFA, a consistent decrease of MUFA, ω7-FA and ω9-FA, and a consistent increase of PUFA (see below). The maternal changes became to a certain extent reflected in the infants, who also showed consistent decreases of 16:0, 18:1ω7, 20:3ω9 and 22:3ω9, and consistent increases of 18:0, 20:0 and 22:0. However, in contrast to the mother, the infants exhibited consistent increases of 14:0, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9, while 24:0 decreased (Figure 1). These results are generally in agreement with earlier studies (mothers ref;[92] infants refs[82,84,87,88]).

The increase of 14:0 in breastfed infants is explained by the unique ability of the breast to synthesize medium chain FA (MCFA) from glucose,[93] but does not occur at the expense of maternal 14:0. This seems to be in contrast with 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9. Very little amounts of these FA are synthesized in the breast,[76,77] and the resulting improvement of the infant's status of

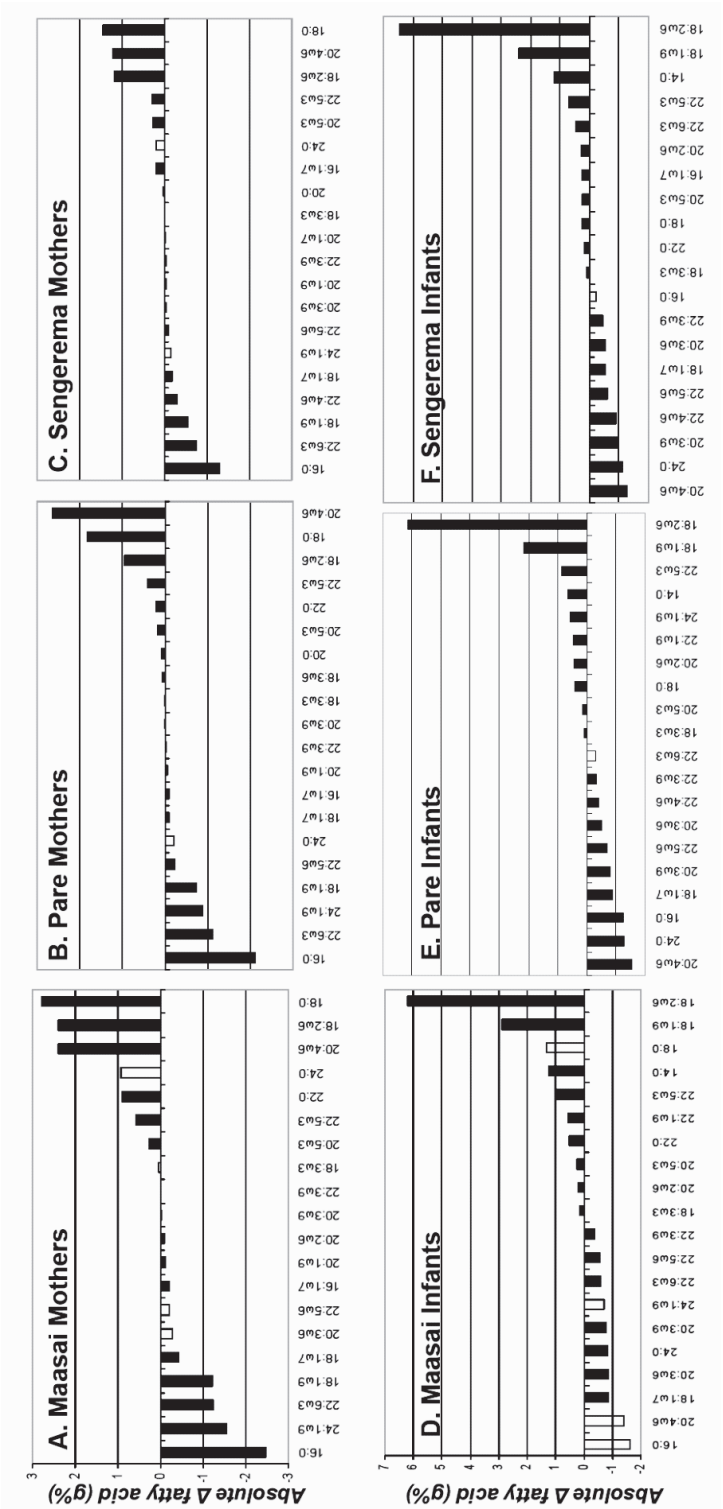

Figure 2. Magnitudes of the differences between selected RBC fatty acids at delivery and 3 months postpartum for Maasai (A), Pare (B) and Sengerema (C) mothers (top) and infants (bottom). A negative (positive) value indicates that the RBC-FA content was lower (higher) at 3 months postpartum than at delivery. Indicated are data for ALA, EPA, DHA, LA, AA, Mead acid (20:3ω9) and 22:3ω9, together with the 13 FA exhibiting the quantitatively highest differences. Closed bars indicate a significant difference between delivery and 3 months postpartum at $p<0.05$; open bars, not significant (see supplemental Tables 1a and 1b); all corrected for type-1 errors.

these FA seems to be at the expense of the maternal status at delivery. The seemingly high mother-to-infant transfer of 18:1ω9 via the breast might be important for growth, and especially that of the brain, which rapidly accretes 18:1ω9 up to at least 4 years of age.[94] This 18:1ω9 accretion might be important to myelin synthesis and may serve as a precursor for the synthesis of nervonic acid (24:1ω9).[49] Like in adults,[42] the infant's transition from a high carbohydrate:fat (transplacental glucose) to a high fat:carbohydrate (breast milk) diet is known to reduce DNL-activity, and consequently the production of 18:1ω9. It is therefore conceivable that the high milk 18:1ω9 ensures a continuing 18:1ω9 supply to the fetus to compensate for the discontinuation of transplacental transfer of 18:1ω9 and the discontinuation of the high DNL of the infant in the third trimester.[49,94,95]

Our data from RBC-FA indicate that hepatic DNL is increased in late pregnancy and decreases from delivery till 3 months PP (Figure 1). We hypothesize that the driving forces for the observed maternal SAFA, ω7-FA and ω9-FA changes from delivery to 3 months PP are likely to be found in the many hormonal changes that accompany the transition from pregnancy to lactation. These include notably the abolition of diminished insulin sensitivity and compensatory hyperinsulinemia of late pregnancy, which are essential to arrive at the states of maternal 'accelerated starvation' and 'facilitated anabolism'[54] that support the developing fetus by the transplacental transfer of important nutrients such as glucose and ketone bodies, and also FA.[54,56-59] After delivery, the lower insulin sensitivity of the third trimester vanishes[54] and insulin levels fall quickly to become comparable to non-pregnant controls within 3 days.[55] This lower insulin sensitivity at late pregnancy may at least partially be driven by hormones such as human placental lactogen (hPL), human placental growth hormone (hPGH)[96,97], sex hormones,[98,99] cortisol,[100] prolactin,[101] leptin[102,103] and cytokines,[104] but each of these might also have their own specific influences on FA metabolism.

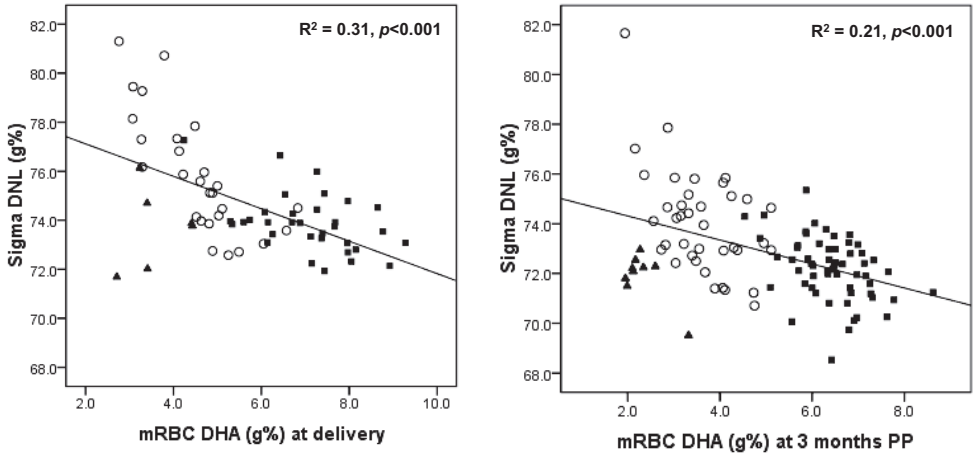

Figure 3. Relations between maternal RBC-DHA at delivery (A) and at 3 months postpartum (B) and the sum of maternal *de novo* synthesized fatty acids. Data are in g%; ▲, Maasai subjects; ○, Pare subjects; ■, Sengerema subjects; R^2, regression coefficient; *p*, significance of the trend line. Sigma DNL, sum of maternal de novo synthesized fatty acids.

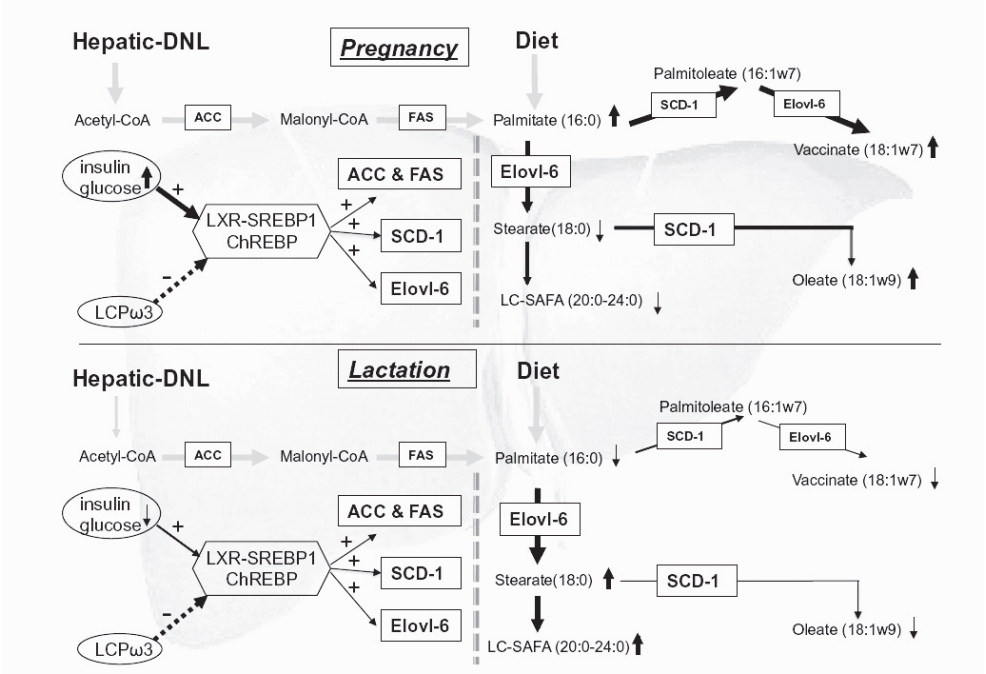

Figure 4. Proposed mechanisms for increased *de novo* lipogenesis (DNL) during pregnancy and the induced synthesis of monounsaturated fatty acids (MUFA) during pregnancy and long-chain saturated fatty acids (LC-SAFA) during lactation. The size of the arrow indicates the magnitude of the pathway or influence; dotted line for long-chain polyunsaturated w3-FA is of unknown magnitude. ACC, acetyl Coenzym-A carboxylase; FAS, fatty acid synthase; SCD-1, stearoyl Coenzym-A desaturase-1; Elovl-6, elongation of very long chain fatty acids family member 6; LXR, liver-X rexeptor; SREBP-1, sterol regulatory binding protein-1; ChREBP, carbohydrate responsive element binding protein.

The decreasing insulin sensitivity and compensatory hyperinsulinemia of late pregnancy result in low DNL[105] together with facilitated lipolysis[106] in maternal adipose tissue, but also with the promotion of DNL in the maternal liver.[41,42] Mechanistically, the lower insulin sensitivity of the liver promotes DNL via activation of LXR and SREBP-1, while the higher maternal glucose in the postprandial state[54] may activate ChREBP.[61,107,108] The outcome is the induction of ACC, FAS, Elovl-6 and SCD-1, which may jointly augment synthesis of 16:0 and 18:0 and their conversion to 16:1ω7, 18:1ω7 and 18:1ω9[61-63] in the liver. The major SAFA produced in the liver is 18:0, rather than 16:0.[109] Our data indeed showed higher DNL and SCD indices, but not higher Elovl-6 activity indices, at delivery compared to 3 months PP (Figure 1). These coincided with post-delivery decreases of DNL-FA in maternal RBC, with the exception of 18:0. The paradoxical increase of 18:0 suggests that the postpartum DNL and SCD-1 activity declines are more pronounced compared with the decline of Elovl-6 activity. Comparably, Ghebremeskel et al.[90] showed higher 16:0, 16:1ω7, 18:1ω9, but lower 18:0 in pregnant women compared to non-pregnant counterparts, supporting high DNL activity, but an even higher SCD activity in pregnancy, resulting in a high 18:1ω9/18:0 ratio. Interestingly,

the 18:0 increase also took place in the infant, which suggests that the post-delivery changing maternal SCD-1 and Elovl-6 activities might be driving forces for 18:0 transfer to the infant via the milk. Since Elovl-6 activity is positively associated with insulin levels,[63] the apparent postpartum increase in Elovl-6 activity is more likely to reflect a decrease in SCD activity, resulting in increased substrate availability for Elovl-6, rather than an increase in Elovl-6 activity *per se* **(Figure 4)**. Finally, as mentioned above, the decreasing insulin sensitivity in late pregnancy is also associated with high adipose tissue lipolytic activity.[106] How this lipolytic activity affects the RBC-FA composition might be complex, since the natural adipose tissue FA abundance is 16:0>18:1ω9>LA>18:0, while the selectivity of FA release occurs in sequence 16:1ω7>16:0>14:0>18:1ω7>LA>18:1ω9>18:0.[110]

Despite the high intakes of 16:0 by Maasai compared to Sengerema, as confirmed by their milk and RBC-FA, there is lower maternal RBC-ΣDNL (Figure 2), 18:0, 16:1ω7 and 18:1ω9 and higher RBC-16:0 with increasing DHA status (Supplemental Figure 1) in the order Maasai<Pare<Sengerema; while the magnitudes of the PP decreases in ΣDNL, 16:0, 16:1ω7 and 18:1ω9, and increases in 18:0 seemed to occur in the order Maasai>Pare>Sengerema (Figure 2**)**, which is the inverse order of increasing fish intake and DHA status. We suggest that these observations might be explained by suppression of SCD[64] and Elovl-6[63] activities by DHA through its influence on SREBP-1 and ChREBP (Figure 4).[61,107,108] The rationale for the suppression of SCD[64] and Elovl-6[63] by DHA might lie in the importance of the ratio between saturated and unsaturated FA, which is known to be of great importance in membrane functionality, such as e.g. reflected by its fluidity, permeability, elasticity and PL flip-flop.[111-113] This ratio is under tight control and its regulation has been suggested to be the primary role of SREBP.[114,115] Incorporation of DHA in membranes has functional effects. For instance, because of the mutual aversion of DHA and cholesterol, incorporation of DHA into a membrane causes formation of highly disordered domains of DHA-containing PL, that segregate from the highly ordered lipid rafts composed of cholesterol and sphingomyelin.[112,113] The present study suggests that DHA suppresses hepatic DNL in late pregnancy. The mechanism may be by improvement of insulin sensitivity,[116] since hepatic DNL in late pregnancy finds its origin in diminished insulin sensitivity.[61,107,108] The influence of fish oil FA on insulin sensitivity and insulin resistance is, however, controversial,[117] possibly because of the many underlying mechanisms that may cause impaired insulin sensitivity. It is possible that the influence of LCPω3 on insulin sensitivity might best be investigated in late pregnancy, which is a state of physiologically reduced insulin sensitivity.

Taken together, the high SAFA and ω7- and ω9-FA in late pregnancy are likely to reflect the mobilization of lipogenesis products that were accumulated in the maternal body during the period of higher insulin sensitivity in early pregnancy,[59] together with the newly formed products of hepatic DNL and SCD activity. Both adipose tissue lipolysis and hepatic DNL in the end of pregnancy are likely to be derived from the well known state of reduced insulin sensitivity and compensatory hyperinsulinemia. We have previously shown that the resulting higher maternal SAFA, ω7-FA and ω9-FA status causes a relative, rather than absolute, EFA deficiency at the end of gestation and that this process occurs more profoundly when the decreased insulin sensitivity of late pregnancy turns into

insulin resistance such as in maternal obesity,[69] diabetes mellitus type 2,[118,119] gestational diabetes mellitus (GDM)[15,120,121] and preeclampsia.[50] The finding of insulin resistance and hyperinsulinemia in large-for-gestational-age infants[122] supports similar effects in the infant. A high DHA status may, by virtue of its DNL-suppressing effect, be of benefit in the prevention of obstetric complications in which insulin resistance and impaired glucose homeostasis play central roles.

ω6 and ω3 fatty acids

The increase of maternal ω6-FA from delivery to 3 months PP was notably on account of LA and AA, while there was a decrease of 22:5ω6. These observations are in line with those of Otto et al.[81] LA and AA increases can partly be explained by the decreases of the *de novo* synthesized ω7-FA and ω9-FA (see above), since RBC-FA are expressed in g%. Secondly, as mentioned above, FADS2 and FADS1 activities are positively associated with insulin levels, but negatively with glucose and DHA levels.[46,48,64] In subjects with impaired glucose tolerance, an increase in insulin sensitivity by lifestyle interventions [diet and exercise[67] or diet[45]] consistently resulted in a net decrease of FADS2 activity and a net increase of FADS1 activity. In line with this observation, our RBC-FA data showed a decrease of FADS2 activity and also suggested an increase of FADS1 activity. The apparent increase in FADS1 activity is, however, more likely to result from the decrease in FADS2 activity, which is the rate limiting step in the conversion of LA and ALA to their LCP-metabolites. Lower FADS2 activity will cause lower 20:3ω6 and the pooling of ALA, 20:5ω3, 22:5ω3, LA, AA and 22:4ω6, giving rise to an increased AA/20:3ω6 ratio that is not representative for FADS1 activity. Hence the development of lower insulin sensitivity in late pregnancy may, by stimulating both FADS1 and FADS2 activity, augment maternal (and fetal) LCP production. Conversely, the postpartum restoration of insulin sensitivity may result in the observed decrease of FADS2 activity, while the concomitant decrease of FADS1 activity might have been obscured by the decreasing FADS2 activity. On the other hand, increasing maternal ω6 (notably LA and AA) status may have added to some extent to the observed lower DNL during lactation, since ω6 PUFA are known to suppress ChREBP[64] although their influence is less strong than ω3 PUFA.

The final explanation of the increasing maternal ω6-FA is the rerouting of LA and AA from transplacental transfer to incorporation into milk lipids and the discontinued utilization of AA by the placenta for the use in structural lipids[57] and prostaglandin synthesis.[123] Haggarty *et al*.[124] suggested that the placenta has a certain minimal requirement for AA and that AA becomes only transported to the fetus after satisfaction of this requirement. We showed both increasing maternal and infant RBC-LA, suggesting a mother-to-infant LA surge via the milk (Figure 1). During pregnancy the infant accretes little LA, partially because most of the maternally derived LA is not incorporated into structural lipids but returned to the mother.[123,125] A minor part of maternal LA becomes trapped in the infant, partially by conversion into AA.[125,126] The low LA status *in utero* and its rapid increase in the infant after birth might be of importance for the postnatal synthesis of unique LA-carrying ceramides in the skin, which act as barriers to limit water losses. The unidirectional surge of LA via

the milk may, as opposed to the bidirectional LA exchange via the placenta, force the building of a water barrier. Possible explanations for the decrease of infant RBC-AA are the interrupted gain via transplacental transfer and diminishing infant and maternal FADS2 activity (see above).

As reported earlier for ω3-FA,[71,72,81] there was an increase during lactation of maternal RBC-ALA, EPA and 22:5ω3, while DHA and also 22:5ω6 (a marker of DHA status) decreased. These changes are again in line with a decreasing FADS2 (and FADS1) activity that accompanies the restoration of insulin sensitivity. The observed changes in the mother coincided with increases of infant RBC-ALA, EPA and 22:5ω3, while 22:5ω6 decreased as well. The only discrepancy between maternal and infant changes from delivery to 3 months PP was the constancy of infant RBC-DHA in the Pare, and its increase in Sengerema. The increase in maternal EPA and 22:5ω3, and the decrease in DHA, were demonstrated earlier in Danish,[127] Dutch[81] and Australian[128] mothers and emphasize the difficulty by which the last step from 22:5ω3 to DHA proceeds (FADS2), although this step occurs with less difficult in women compared with men.[99] The decreasing magnitudes of the postpartum maternal and infant RBC-22:5ω3 increases in the order Maasai>Pare>Sengerema (Figure 2) suggest the highest DHA synthetic-activities in the populations with the lowest DHA-status. The postpartum maternal DHA losses were accompanied by a seemingly paradoxical decrease of 22:5ω6, which is a marker of DHA deficiency. Also infant RBC-20:3ω9 and 22:3ω9, both markers of EFA deficiency, showed decreases, that occurred concomitant with the decrease of infant RBC-DHA. This suggests that under these conditions, these markers of DHA and EFA status are of little value and are more likely to reflect the PP decreases in maternal and infant DNL, SCD, FADS1 and FADS2 activities, rather than an improvement in the EFA or DHA status *per se*.[92] Finally, the previously suggested[110] selective mobilization of LCP from adipose tissue (in the order EPA>AA>ALA>16:1ω7>DHA>16:0>14:0>18:1 ω7>LA>18:1ω9>18:0) might also contribute to the observed specific RBC-FA changes from delivery to 3 months PP.

CONCLUSION AND IMPLICATIONS

Postdelivery changes in maternal and infant RBC-FA compositions might notably be influenced by the restoration of insulin sensitivity and the maternal DHA status, since DHA is a potent suppressor of ChREBP and SREBP-1, and thereby of DNL and FADS. The changing insulin sensitivity during pregnancy and lactation, together with the influence of the maternal DHA status on the activities of FAS, ACC, SCD, Elovl-6, FADS1 and FADS2, are likely to aim at the transfer to the infant of specific FA, such as 16:0, 18:1ω9, AA and DHA during gestation and 14:0, 18:0, 18:1ω9, LA, AA and DHA during lactation. The postpartum maternal and infant increases of ALA, EPA and 22:5ω3 support the notion that FADS2 is rate limiting in DHA synthesis, especially during lactation. A sufficiently high maternal DHA status might not only be important for structural purposes in e.g. the fetal central nervous system, but also for the modulation of insulin sensitivity and thereby maternal hepatic DNL in late pregnancy. DHA in pregnancy may therefore be of importance in the prevention of obstetric complications in which insulin resistance and impaired glucose homeostasis play central

roles. Taken together, we conclude that perinatal changes in maternal and infant FA status may be strongly driven by changing insulin sensitivity and DHA status. To test our hypothesis any future study would need to correlate insulin sensitivity, actual enzymatic activity and use stable isotopically labeled RBC-FA, preferably in a longitudinal study.

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Supplemental Table 1a. Maternal erythrocyte fatty acids at delivery and at 3 months postpartum for Maasai, Pare and Sengerema[1,2]

	Maasai (no fish)		Pare (medium fish)		Sengerema (high fish)		M-P	P-S	S-M	M-P	P-S	S-M
	Delivery	3 months PP	Delivery	3 months PP	Delivery	3 months PP	delivery			3 months PP		
	g/100 g fatty acids (g%)											
Mothers, n	6	9	27	38	34	60						
14:0	0.48 (0.37-0.52)	0.49 (0.05-0.95)	0.43 (0.27-0.66)	0.44 (0.04-1.60)	0.39 (0.27-0.84)	0.40 (0.27-1.34)						
16:0	26.5 (25.7-28.7)	24.1 (22.9-24.6)**	28.1 (24.9-31.8)	26.0 (24.7-29.1)***	28.4 (26.4-30.6)	27.1 (23.7-30.0)***			*	*	*	*
18:0	16.4 (15.1-16.8)	19.2 (18.0-20.3)**	16.7 (15.2-19.2)	18.5 (16.6-25.5)***	16.2 (14.9-18.0)	17.7 (15.8-21.7)***					*	*
20:0	0.46 (0.42-0.86)	0.58 (0.51-0.72)	0.50 (0.41-0.64)	0.58 (0.39-1.04)**	0.53 (0.40-0.81)	0.56 (0.42-0.94)*						
22:0	2.07 (1.71-2.50)	2.98 (2.30-3.99)**	2.27 (1.78-2.88)	2.48 (1.70-4.39)*	2.15 (1.71-2.88)	2.27 (1.86-2.82)				*	*	*
24:0	5.85 (3.29-7.22)	6.77 (6.31-8.15)	6.75 (5.53-8.44)	6.56 (0.82-8.36)	6.04 (4.30-7.60)	6.25 (5.13-7.99)		*				*
SAFA	52.0 (49.5-53.7)	54.4 (52.3-56.5)*	54.3 (51.0-60.2)	54.8 (51.9-58.4)	53.7 (51.6-57.5)	54.4 (49.0-57.7)	*		*			
LCSAFA	51.5 (48.9-53.2)	53.7 (51.8-56.1)*	53.9 (50.6-59.8)	54.6 (51.6-57.5)	53.4 (51.2-57.1)	54.0 (48.6-57.3)	*		*	*		
16:1ω7	0.64 (0.54-0.77)	0.43 (0.30-0.66)*	0.44 (0.06-0.69)	0.34 (0.05-0.75)*	0.11 (0.06-0.23)	0.31 (0.06-0.55)***	*	*	*	*		*
18:1ω7	1.56 (1.24-1.86)	1.13 (0.85-1.48)	1.46 (1.12-2.57)	1.36 (0.90-3.10)*	1.56 (1.03-2.30)	1.38 (1.10-1.99)***				*		*
20:1ω7	0.04 (0.03-0.10)	0.03 (0.02-0.06)	0.04 (0.01-0.10)	0.04 (0.00-0.15)	0.06 (0.02-0.11)	0.04 (0.01-0.21)**						*
Σω7	2.26 (1.87-2.60)	1.57 (1.33-1.98)*	1.99 (1.30-3.16)	1.74 (1.08-3.34)*	1.76 (1.27-2.49)	1.71 (1.29-2.59)		*	*			
18:1ω9	12.9 (11.6-14.8)	11.7 (10.0-13.2)*	12.3 (10.7-14.6)	11.5 (9.20-13.4)*	11.6 (9.91-14.0)	11.0 (9.52-13.5)**		*	*		*	
20:1ω9	0.24 (0.19-0.47)	0.13 (0.12-0.23)**	0.21 (0.12-0.32)	0.17 (0.09-0.43)*	0.19 (0.11-0.49)	0.14 (0.07-0.32)***				*	*	
20:3ω9	0.36 (0.26-0.61)	0.33 (0.23-0.51)	0.23 (0.12-0.62)	0.24 (0.07-0.90)	0.23 (0.14-0.65)	0.17 (0.10-2.13)**			*		*	*
22:1ω9	0.11 (0.07-0.17)	0.09 (0.07-0.11)	0.08 (0.05-0.14)	0.08 (0.01-0.42)	0.10 (0.06-0.13)	0.08 (0.05-0.16)**						
22:3ω9	0.14 (0.06-0.40)	0.13 (0.07-0.16)	0.09 (0.03-0.21)	0.07 (0.02-0.57)	0.06 (0.00-0.14)	0.02 (0.00-0.39)***			*	*	*	*
24:1ω9	5.43 (4.52-6.85)	3.89 (3.32-4.32)**	5.68 (4.78-7.17)	4.80 (0.61-9.03)***	4.45 (3.26-5.11)	4.30 (3.56-5.52)		*	*	*	*	*
Σω9	19.9 (17.5-22.2)	16.0 (14.5-18.0)**	18.8 (16.3-21.9)	17.0 (14.3-21.7)***	18.2 (15.1-20.6)	16.2 (14.1-18.8)***			*		*	
MUFA	21.4 (18.9-24.0)	17.1 (15.5-19.5)**	20.3 (17.9-24.6)	18.2 (15.6-23.6)***	18.2 (15.3-21.0)	17.4 (15.2-20.6)**		*	*	*	*	
ΣDNL(adult)	73.8 (71.7-76.1)	72.2 (69.5-73.0)	75.4 (72.6-81.3)	74.0 (70.7-81.6)***	73.9 (71.9-77.2)	72.3 (68.5-75.3)***		*		*	*	

Values are median (range); data are in g%.

Abbreviations: PP, postpartum; FA, fatty acids; SAFA, saturated FA; LCSAFA, long-chain SAFA (i.e ≥16:0); MUFA, monounsaturated FA; DNL, de novo lipid synthesis; ΣDNL (adult) sum of SAFA, ω7 and ω9.

[1], Statistics: significant differences between delivery and 3 months PP are indicated by *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$

[2], Significant between-group differences (M, Maasai; P, Pare; S, Sengerema) at *, $p<0.05$

Supplemental Table 1b. Maternal enzyme activity ratio indices at delivery and at 3 months postpartum for Maasai, Pare and Sengerema[1]

	Maasai (no fish)		Pare (medium fish)		Sengerema (high fish)	
	Delivery	3 months PP	Delivery	3 months PP	Delivery	3 months PP
	g/100 g fatty acids (g%)					
Mothers, n	6	9	27	38	34	60
DNL (16:0/18:2ω6)	3.34 (2.69-3.75)	2.29 (2.13-3.01)**	2.80 (2.03-4.27)	2.46 (1.84-3.20)***	3.09 (2.27-4.38)	2.56 (1.68-3.59)***
SCD (16:1ω7/16:0)	0.025 (0.02-0.03)	0.018 (0.01-0.03)*	0.015 (0.00-0.02)	0.013 (0.00-0.03)	0.004 (0.00-0.01)	0.011 (0.00-0.02)*
SCD (18:1ω7/16:0)	0.057 (0.05-0.07)	0.047 (0.04-0.06)**	0.052 (0.04-0.09)	0.051 (0.03-0.12)	0.054 (0.04-0.08)	0.051 (0.04-0.08)*
SCD (18:1ω9/18:0)	0.09 (0.08-0.12)	0.06 (0.04-0.08)	0.09 (0.06-0.17)	0.07 (0.05-0.12)***	0.10 (0.07-0.14)	0.07 (0.06-0.12)*
D5D (20:4ω6/20:3ω6)	7.09 (4.19-9.64)	9.11 (7.59-16.5)**	7.18 (5.08-10.6)	8.13 (5.53-11.2)**	8.00 (6.63-11.5)	9.06 (5.43-12.1)***
D6D (22:6ω3/20:5ω3)	20.7 (11.1-26.2)	4.93 (2.61-11.9)**	21.1 (10.3-96.2)	8.45 (3.82-21.6)***	20.9 (7.86-44.1)	9.70 (4.05-40.4)***
D6D (22:6ω3/22:5ω3)	2.65 (1.49-2.95)	0.91 (0.66-1.78)**	3.19 (1.84-4.71)	1.97 (1.15-3.36)***	4.17 (2.73-5.64)	3.34 (2.28-4.56)***
D6D (20:3ω6/18:2ω6)	0.26 (0.20-0.32)	0.18 (0.14-0.20)*	0.16 (0.10-0.25)	0.16 (0.11-0.25)	0.17 (0.08-0.24)	0.15 (0.09-0.34)***
D6D (22:5ω6/20:4ω6)	0.08 (0.05-0.15)	0.06 (0.04-0.07)	0.09 (0.06-0.17)	0.06 (0.04-0.09)***	0.07 (0.04-0.13)	0.06 (0.04-0.10)***
D6D (22:5ω6/22:4ω6)	0.49 (0.31-0.54)	0.34 (0.23-0.37)*	0.37 (0.16-0.52)	0.30 (0.22-0.55)***	0.36 (0.25-0.60)	0.37 (0.28-0.49)
Elovl-6 (18:0/16:0)	0.62 (0.53-0.64)	0.81 (0.73-0.88)***	0.58 (0.50-0.76)	0.70 (0.62-0.99)***	0.57 (0.50-0.64)	0.65 (0.57-0.88)***
Elovl-6 (18:1ω7/16:1ω7)	2.36 (1.93-3.44)	2.76 (1.60-3.44)	4.11 (1.68-20.2)	3.91 (1.93-27.4)	14.6 (4.90-31.9)	4.61 (2.51-21.5)***

Values are median (range); data are in g/g.

Abbreviations: PP, postpartum; DNL, de novo lipid synthesis; SCD, stearoyl Co-A desaturase or Δ9-desaturase; D5D, Δ5-desaturase; D6D, Δ6-desaturase; Elovl-6, elongation of long chained fatty acids family member 6.

[1], Statistics: significant differences between delivery and 3 months PP are indicated by *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

Supplemental Table 2a. Infant erythrocyte fatty acids at delivery and at 3 months postpartum for Maasai. Pare and Sengerema[1,2]

	Maasai (no fish)				Pare (medium fish)				Sengerema (high fish)				M-P	P-S	S-M	M-P	P-S	S-M
	Delivery		3 months PP		Delivery		3 months PP		Delivery		3 months PP		delivery			3 months PP		
	g/100 g fatty acids (g%)																	
Infants. n	8		8		29		38		36		61							
14:0	0.47	(0.41-1.90)	1.71	(0.78-1.96)*	0.43	(0.32-0.84)	1.09	(0.51-2.79)***	0.44	(0.01-0.69)	1.64	(0.61-4.91)***					*	
16:0	27.4	(26.3-28.8)	25.8	(23.7-32.9)	26.8	(24.7-30.2)	25.6	(23.0-29.5)***	26.3	(25.0-29.5)	26.1	(22.9-29.3)			*			
18:0	16.8	(15.6-18.1)	18.1	(12.0-19.9)	18.4	(16.4-21.6)	18.8	(17.1-32.3)**	17.8	(15.4-19.1)	18.1	(16.4-23.6)**	*	*			*	
20:0	0.74	(0.59-0.82)	0.78	(0.37-0.82)	0.75	(0.52-2.33)	0.82	(0.54-1.87)**	0.74	(0.52-0.92)	0.80	(0.51-2.03)***						
22:0	1.53	(1.43-1.78)	2.03	(1.08-2.30)*	1.73	(1.57-2.09)	2.05	(1.48-2.89)***	1.73	(1.43-2.08)	1.91	(1.55-2.48)***	*		*		*	
24:0	5.72	(5.17-6.54)	4.90	(2.08-5.38)**	6.74	(5.57-7.67)	5.47	(3.47-6.41)***	6.08	(4.84-6.96)	4.95	(3.48-6.10)***	*	*			*	
SAFA	52.4	(50.4-56.6)	52.2	(50.5-54.6)	54.5	(52.1-63.4)	53.9	(51.3-67.2)	53.0	(51.4-56.6)	53.9	(51.2-58.9)**	*	*				
LCSAFA	52.0	(49.7-54.7)	50.9	(48.5-53.0)	54.0	(51.7-62.5)	52.4	(50.3-65.6)**	52.6	(51.1-56.2)	52.1	(49.9-56.3)**	*	*				
16:1ω7	0.63	(0.53-1.39)	0.43	(0.31-2.38)	0.44	(0.32-0.64)	0.29	(0.16-2.10)***	0.09	(0.04-0.23)	0.35	(0.09-2.44)***	*	*	*			
18:1ω7	2.10	(1.74-2.91)	1.24	(0.96-2.38)*	2.10	(1.62-2.50)	1.22	(0.06-1.57)***	1.99	(1.56-2.73)	1.44	(1.09-2.01)***					*	
20:1ω7	0.06	(0.02-0.16)	0.12	(0.07-0.31)*	0.05	(0.01-0.08)	0.07	(0.01-0.33)*	0.06	(0.03-0.87)	0.10	(0.03-0.49)***					*	
Σω7	2.71	(2.62-4.38)	1.83	(1.45-4.89)*	2.59	(2.07-3.20)	1.69	(0.43-3.25)***	2.14	(1.67-3.20)	1.90	(1.29-3.77)***		*	*		*	
18:1ω9	9.35	(8.28-10.7)	12.2	(11.3-25.1)**	7.86	(6.76-9.23)	10.0	(7.76-14.9)***	8.34	(6.64-13.8)	10.7	(8.62-13.9)***	*	*	*	*	*	*
20:1ω9	0.13	(0.09-0.27)	0.26	(0.21-0.39)**	0.10	(0.06-0.14)	0.25	(0.13-0.81)***	0.11	(0.08-0.15)	0.19	(0.08-0.30)***	*	*	*		*	*
20:3ω9	1.07	(0.81-2.70)	0.29	(0.17-0.77)**	0.99	(0.55-1.42)	0.21	(0.02-1.39)***	1.09	(0.57-2.19)	0.14	(0.04-0.36)***					*	*
22:1ω9	0.09	(0.05-0.13)	0.66	(0.27-1.49)**	0.06	(0.02-0.15)	0.54	(0.15-3.05)***	0.06	(0.05-0.09)	0.25	(0.10-0.74)***	*		*		*	*
22:3ω9	0.47	(0.33-0.72)	0.13	(0.04-0.43)**	0.46	(0.23-0.70)	0.12	(0.00-0.86)***	0.50	(0.03-0.85)	0.04	(0.00-0.16)***					*	*
24:1ω9	4.29	(3.49-5.28)	3.57	(1.61-4.94)	3.90	(3.22-4.76)	4.47	(1.18-6.05)***	3.91	(3.08-4.46)	3.76	(2.67-4.92)					*	
Σω9	15.4	(13.4-19.7)	18.5	(16.7-27.6)*	13.3	(11.6-15.1)	16.1	(11.8-22.9)***	15.4	(13.8-19.4)	15.7	(12.2-20.0)	*	*		*		*
MUFA	16.6	(14.8-20.7)	19.5	(18.0-32.2)**	14.4	(12.5-16.4)	17.8	(13.1-25.0)***	14.7	(12.9-19.5)	17.0	(14.2-20.2)***	*		*	*		*
ΣDNL (adult)	71.1	(69.3-74.7)	73.3	(69.6-83.0)	70.8	(67.0-77.3)	71.5	(67.6-82.0)*	70.4	(68.8-75.2)	71.5	(68.3-77.0)						
ΣDNL (infant)	61.5	(59.9-63.1)	58.5	(55.9-61.3)**	62.3	(59.7-69.7)	60.3	(57.7-72.7)**	61.5	(59.8-64.6)	58.8	(56.2-62.8)***				*	*	

Values are median (range); data are in g%.

Abbreviations: PP, postpartum; FA, fatty acids; SAFA, saturated FA; LCSAFA, long-chain SAFA (i.e ≥16:0); MUFA, monounsaturated FA; DNL, de novo lipid synthesis; ΣDNL (adult) sum of SAFA, ω7 and ω9.

[1], Statistics: significant differences between delivery and 3 months PP are indicated by *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$

[2], Significant between-group differences (M, Maasai; P, Pare; S, Sengerema) at *, $p<0.05$

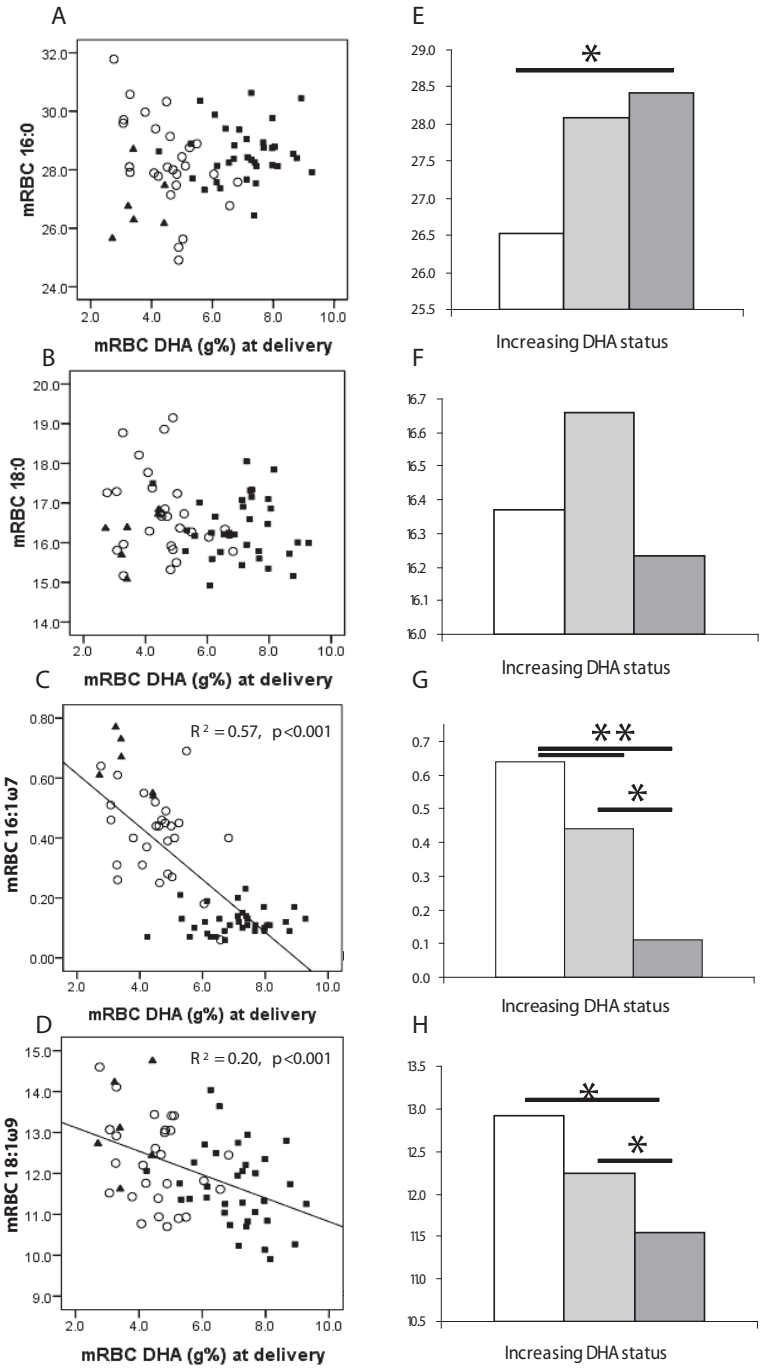
Supplemental Table 2b. Infant enzyme activity ratio indices at delivery and at 3 months postpartum for Maasai, Pare and Sengerema[1]

	Maasai (no fish)				Pare (medium fish)				Sengerema (high fish)			
	Delivery		3 months PP		Delivery		3 months PP		Delivery		3 months PP	
	g/100 g fatty acids (g%)											
Infants, n	8		8		29		38		36		61	
DNL (16:0/18:2ω6)	8.85	(6.38-11.3)	3.16	(2.07-3.53)***	8.55	(6.38-12.7)	2.66	(2.12-3.95)***	7.98	(5.02-13.6)	2.62	(1.53-4.28)***
SCD (16:1ω7/16:0)	0.023	(0.02-0.05)	0.016	(0.01-0.07)	0.017	(0.01-0.02)	0.011	(0.01-0.09)***	0.003	(0.00-0.01)	0.014	(0.00-0.09)***
SCD (18:1ω7/16:0)	0.076	(0.06-0.11)	0.050	(0.04-0.07)**	0.078	(0.06-0.09)	0.049	(0.00-0.06)***	0.076	(0.06-0.10)	0.054	(0.04-0.08)***
SCD (18:1ω9/18:0)	0.12	(0.10-0.19)	0.07	(0.05-0.20)	0.11	(0.08-0.14)	0.06	(0.00-0.09)***	0.11	(0.09-0.15)	0.08	(0.06-0.11)***
D5D (20:4ω6/20:3ω6)	6.56	(5.36-9.49)	9.67	(8.51-15.6)**	7.01	(3.95-8.83)	8.44	(6.25-15.0)***	7.40	(5.56-8.58)	8.84	(5.46-14.3)***
D6D (22:6ω3/20:5ω3)	45.2	(17.0-74.0)	12.8	(5.20-25.7)**	42.9	(21.7-509)	17.9	(9.05-77.0)***	36.2	(7.66-105)	14.5	(6.24-28.9)***
D6D (22:6ω3/22:5ω3)	11.6	(6.97-13.3)	2.34	(1.76-4.38)**	12.4	(9.12-26.7)	3.56	(2.83-5.88)***	12.3	(3.51-16.8)	5.65	(3.73-7.07)***
D6D (20:3ω6/18:2ω6)	0.81	(0.60-0.91)	0.18	(0.07-0.23)***	0.71	(0.53-1.05)	0.19	(0.10-0.31)***	0.65	(0.28-1.20)	0.17	(0.07-0.29)***
D6D (22:5ω6/20:4ω6)	0.11	(0.08-0.16)	0.08	(0.04-0.09)**	0.10	(0.08-0.14)	0.07	(0.04-0.11)***	0.08	(0.04-0.15)	0.05	(0.04-0.09)***
D6D (22:5ω6/22:4ω6)	0.64	(0.55-0.73)	0.46	(0.34-0.58)**	0.53	(0.43-0.69)	0.37	(0.25-0.55)***	0.49	(0.33-0.78)	0.38	(0.32-0.56)***
Elovl-6 (18:0/16:0)	0.62	(0.56-0.66)	0.73	(0.36-0.83)**	0.69	(0.61-0.75)	0.74	(0.65-1.22)***	0.67	(0.52-0.75)	0.69	(0.58-0.91)**
Elovl-6 (18:1ω7/16:1ω7)	3.22	(2.09-3.91)	2.83	(1.00-4.13)	4.37	(3.30-7.47)	4.08	(0.38-9.12)	22.5	(8.83-49.5)	4.26	(1.47-12.6)***

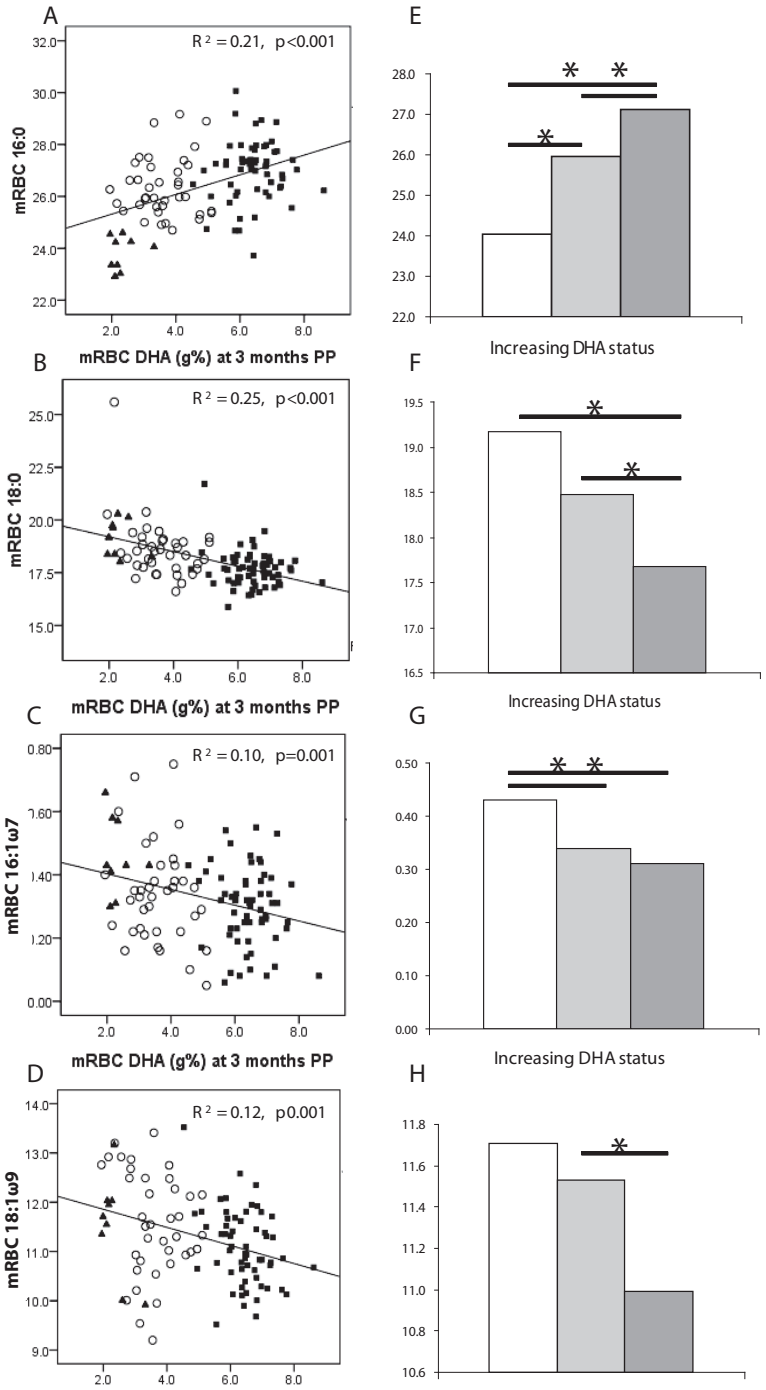
Values are median (range); data are in g/g.

Abbreviations: PP, postpartum; DNL, de novo lipid synthesis; SCD, stearoyl Co-A desaturase or Δ9-desaturase; D5D, Δ5-desaturase; D6D, Δ6-desaturase; Elovl-6, elongation of long chained fatty acids family member 6.

[1], Statistics: significant differences between delivery and 3 months PP are indicated by *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.



Supplemental Figure 1. Relation between maternal RBC-DHA at delivery and maternal RBC-16:0 (A,E), 18:0 (B,F), 16:1ω7 (C,G) and 18:1ω9 (D,H). Data are in g%. ▲, Maasai subjects; ○, Pare subjects; ■, Sengerema subjects; R^2, regression coefficient; *p*, significance of the trendline; *, significantly different between groups. The bars in the right panels represent the medians for RBC-16:0, 18:0, 16:1ω7 and 18:1ω9, respectively, for the Maasai (dotted bars), Pare (light grey) and Sengerema (dark grey).



Supplemental Figure 2. Relation between maternal RBC-DHA at 3 months postpartum and maternal RBC-16:0 (A,E), 18:0 (B,F), 16:1ω7 (C,G) and 18:1ω9 (D,H). For other legend see supplemental figure 1.

CHAPTER 7.3

Differences in preterm and term milk fatty acid compositions may be caused by the different hormonal milieu of early parturition

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ABSTRACT

Introduction. The hormonal milieus of pregnancy and lactation are driving forces of nutrient fluxes supporting infant growth and development. The decrease of insulin sensitivity with compensatory hyperinsulinemia with advancing gestation, causes adipose tissue lipolysis and hepatic *de novo* lipogenesis (DNL).

Subjects & Methods. We compared fatty acid (FA) contents and FA-indices for enzyme activities between preterm (28-36 weeks) and term (37-42) milks, and between colostrum (2-5 days), transitional (6-15) and mature (16-56) milks. We interpreted FA differences between preterm and term milks, and their changes with lactation, in terms of the well known decrease of insulin sensitivity during gestation and its subsequent postpartum restoration, respectively.

Results. Compared with term colostrum, preterm colostrum contained higher indices of DNL in the breast (DNL-breast) and medium-chain-saturated-FA (MCSAFA), and lower DNL-liver and monounsaturated-FA (MUFA). Preterm milk also had higher docosahexaenoic acid (DHA) in colostrum and transitional milk and higher arachidonic acid (AA) in mature milk. Most preterm-term differences vanished with advancing lactation. In both preterm and term milks, DNL-breast and MCSAFA increased with advancing lactation, while DNL-liver, MUFA, long-chain-SAFA and AA decreased. DHA decreased in term milk. MUFA was inversely related to MCSAFA in all samples, correlated inversely with PUFA in colostrum and transitional milks, but positively in mature milk. MCSAFA correlated inversely with PUFA in mature milk.

Conclusion. Higher maternal insulin sensitivity at preterm birth may be the cause of lower MUFA (a proxy for DNL-liver) and higher MCSAFA (a proxy for DNL-breast) in preterm colostrum, compared with term colostrum. Restoring insulin sensitivity after delivery may be an important driving force for milk FA-changes in early lactation.

INTRODUCTION

Milk is composed of fatty acids (FA) from different origins.[129] Medium chain saturated-FA (MCSAFA; ≤14:0) derive mostly from endogenous mammary gland *de novo* lipogenesis (DNL). Long chain saturated-FA (LCSAFA; ≥16:0) and monounsaturated- (MUFA) derive mainly from the circulation, since their *de novo* synthesis is limited within the breast.[76,77,129] Long-chain polyunsaturated-FA (LCP), i.e. the long-chain metabolites of the parent essential-FA (EFA) linoleic (LA) and α-linolenic (ALA) acids, also derive from the circulation, since they cannot be synthesized *de novo*, while the conversion of polyunsaturated-FA (PUFA) to LCP within the mammary gland is limited.[78,130] The milk FA composition results from some combination of FA uptake from the triglycerides (TG) in circulating chylomicrons and VLDL by lipoprotein lipase (LPL),[80] FA uptake from circulating albumin-bound free-FA (FFA)[129] and from endogenous DNL from glucose in the breast. Mammary gland FA uptake from the circulation seems rather unspecific.[78,131] All together, it was estimated that 10-12% of milk FA derive from DNL in the mammary gland, that 60% derive from maternal stores, including hepatic and adipose tissue DNL, and that 29% originate 'directly' from the diet, either from chylomicrons ($t^{½}$ = 0.1-1 hour) or from chylomicron remnants following the incorporation of their FA into VLDL ($t^{½}$ = 1-3 hours) in the liver[80,132] About 30% of the EFA are considered to originate 'directly' from the diet and about 70% from maternal depots.[78] The milk contents of e.g. MCSAFA and LCP are also highly dependent on the carbohydrate and fish contents of the diet, respectively.[133,134] However, many other factors are known to influence the milk FA composition.[135] Some of these are not directly related to diet, but associated with the duration of lactation or with the gestational age at delivery.

Several studies investigated the mechanisms underlying milk FA changes with advancing lactation and milk FA differences between preterm and term milks. It has been concluded that MCSAFA synthesis in the mammary gland is triggered by parturition, independent of length of gestation, and that MCSAFA synthesis increases until week 2-4 of lactation.[136-138] LPL-activity in mammary tissue increases 1-2 days before parturition[139] and continues to increase during lactation.[138-141] Prolonged gestation and prolonged lactation may cause depletion of maternal LCP stores[79] and thereby cause lower LCP in preterm, compared to term milk, and a decrease of milk-LCP with advancing gestation, respectively. Differences between preterm and term human milks cannot be explained on a molecular basis, since no differences in TG-structure,[142] PL-classes,[143] percentages PL from total lipid*[144] or fat globule surface area[145] were noted. Conversely, the percentage LCP-rich phospholipids (PL) decreases with advancing lactation,[137,143,144,146] while TG increases from 96% in colostrum to 99.5% in mature milk. All of these changes coincide with an increase of the average fat globule diameter,[145] a decline of the cell concentration[147] and a decrease of the relative total fat globule surface area.[147,148]

Taken together, some combination of these observed changes result in the quite consistently found higher LCP, notably arachidonic (AA) and docosahexaenoic (DHA) acids,[149-153] and

* The higher percentage phospholipids in (very) preterm compared to term milk as noted in the classic study of Bitman et al.,[143] is influenced by the movement of one data point for term milk in one publication[154] to another location in the next[143], without explanation. We therefore refer to the more recent study from Shoi et al.[144]

MCSAFA,[149,151,154-157] and lower MUFA[151,154-156,158] in early (≤10 days) preterm compared to early term milk. Likewise, they might explain the consistently observed increase of the milk fat content[154,159,160] and the concurrent increase of MCSAFA, and the decrease of MUFA and LCP from colostrum to mature milk.[137,161-163] The driving forces of these changes might be found in the hormonal milieus of pregnancy and lactation that aim at optimal support of fetal and newborn growth and fat accretion through the regulation of maternal glucose and lipid fluxes across the placenta and mammary gland, respectively. The second half of pregnancy is characterized by a physiological state of reduced maternal insulin sensitivity that is followed by compensatory hyperinsulinemia and progresses towards full term gestation.[54,56] This state of reduced insulin sensitivity/compensatory hyperinsulinemia causes increased adipose tissue lipolytic activity and increased *de novo* lipogenesis (DNL) that jointly result in the hyperlipidemia of pregnancy.[54,56] Hudgins et al.[164] showed that the great majority of DNL takes places in the liver, rather than in adipose tissue. The increased DNL activity becomes e.g. reflected in the higher SAFA and MUFA contents in maternal plasma-PL and erythrocytes (RBC) in late, compared to early, gestation.[71-74] The reduced insulin sensitivity/hyperinsulinemia of late gestation, however, vanishes shortly after pregnancy termination.[55] From this perspective, we recently suggested[165] that the consistently observed postpartum decreases of DNL-derived FA in maternal and infant RBC that occur between delivery and 3 months postpartum, might be the result of the concurrent restoration of insulin sensitivity. However, this hypothesis had not yet been applied to the differences between preterm and term milk FA compositions and the changes observed in the milk FA composition after delivery.

Compared to a preterm delivery, the lower maternal insulin sensitivity after a term delivery[54] is likely to result in a higher adipose tissue lipolytic activity and hepatic DNL-activity, resulting in higher circulating FFA and VLDL, respectively, and a higher relative contribution of FA from hepatic DNL-activity to the VLDL-TG. Consequently, we hypothesize that (in women with similar diets and adiposity) i) the lower maternal insulin sensitivity after term delivery underlies a higher LCSAFA and MUFA, and lower MCSAFA, in term colostrum compared to preterm colostrum, secondary to the competition between FA from the circulation (LCSAFA, MUFA, PUFA) and those from endogenous mammary gland synthesis (MCSAFA). Similarly, we hypothesize that, ii) despite the increase in mammary gland LPL-activity with advancing lactation,[138-141] the restoring insulin sensitivity after delivery also underlies a decreasing milk LCSAFA and MUFA, and increasing milk MCSAFA with advancing lactation. To additionally test the suggested depletion of maternal LCP stores with advancing gestation and lactation,[79] we chose to investigate the preterm and term milk FA compositions of Tanzanian women with very high fish intakes, resulting in a high DHA status. We have previously reported that these women exhibit no decreases in their RBC-DHA contents during gestation.[166] At delivery, their infants had lower RBC-DHA contents compared to their mothers,[53] suggesting that under these conditions DHA transfer from mother-to-infant becomes rather inhibited than stimulated. DHA depletion during pregnancy is therefore unlikely and we consequently expected no appreciable differences in the DHA contents of preterm and term

colostrum in these women.

MATERIALS AND METHODS

Study design

In a cross sectional study we investigated the FA differences between colostrum (by definition: 1-5 days), transitional (6-14 days) and mature breast milk (>14 days). The milk samples derived from a dietary highly homogeneous group of women in Tanzania with high intakes of both carbohydrates and AA and DHA from local tropical freshwater fish.[52] They delivered either prematurely (28-36 weeks of gestation) or at term (37-42 weeks). Due to the limited amount of seasonally available nutrients of the local people, maternal diets are highly homogenous, while neither pregnancy nor lactation are known to be associated with changes in the local dietary customs or intakes. As such, differences in the dietary intakes between women who deliver preterm or term, or at any time-point during lactation, are considered negligible. Similarly, adiposity is rare among rural Africans. Consequently, no difference was observed between the BMI of mothers after preterm or term delivery;[89] making differences in adiposity an unlikely contributor to preterm-term milk differences The study was conducted in the Sengerema hospital, at the southern shore of Lake Victoria in Tanzania. Part of the results was published previously.[89] High dietary fish intakes in this population were confirmed by their milk LCP contents[89] and from the RBC-LCP contents of a population sampled in the same area.[53,166] In the present study, we additionally investigated the FA-sums and FA-indices that might provide information about enzyme activities in FA metabolism at delivery and their changes thereafter. Secondly, we reviewed the literature for the consistency of our findings, and to support or falsify the suggested mechanisms that may drive the observed differences.

Rationale for FA-sums and FA-indices

Lipid synthesis and fluxes might become reflected by the contents of specific milk-FA and their ratios. For instance, hepatic DNL is directed at 16:0 that may subsequently become elongated to 18:0 by the enzyme for 'elongation of very long-chain-FA' (Elovl-6). Both 16:0 and 18:0 may become desaturated by stearoyl-CoA desaturase (SCD) to form 16:1ω7 and 18:1ω9, respectively[62] Subsequent elongation of 16:1ω7 and 18:1ω9 by Elovl-6 will form 18:1ω7 and 20:1ω9, respectively.[63] In contrast to DNL in the liver, DNL in the mammary gland is directed at 14:0 and only small amounts of SAFA are desaturated within the breast.[76,77,129] For instance, only 1% of 14:0 is converted to 14:1ω5. Consequently, one might differentiate between the sum of those milk-FA that derive, at least in part, typically from hepatic DNL (ΣDNL-liver) and those from DNL in the breast (ΣDNL-breast). Upon interpretation of these proxies, it is important to realize that the majority of FA in the ΣDNL-liver derive from adipose tissue stores and/or diet. Hence, ΣDNL-liver, but to a much lesser extent ΣDNL-breast, overestimates the DNL percentages that are obtained from studies with stable isotopes. Δ5-desaturase (D5D) and Δ6-desaturase (D6D) are both involved in EFA-desaturation.[45-48] Since EFA cannot be synthesized *de novo,* they are all ultimately derived from the diet and/or from maternal

stores. Hormone-driven changes in the activities of the many enzymes mentioned above might consequently become reflected by their enzyme product/EFA ratios (DNL activity indices) and alternatively from their product/substrate ratios (SCD, and D5D and D6D activity indices). The actual activity and/or expression of these enzymes have been directly or indirectly related to most of these indices in serum lipids[45,47,65-67,69] and adipose tissue.[70] To our knowledge they have as yet not been systematically applied in studies on the human milk-FA composition.

Hormones influencing FA-enzymatic activities

The activities of hepatic DNL and SCD,[64] but also of D5D and D6D are influenced by glucose,[46,48] insulin[48,61,63] and LCPω3.[45,64] Since dietary LCPω3 intakes were similar in our preterm and term subjects, we focus our discussion on the possible influence of insulin sensitivity. However, other hormones such as human placental lactogen (hPL), human placental growth hormone (hPGH),[96,97] sex hormones,[98] cortisol,[167] prolactin,[101] leptin[102] and cytokines[104,168] likewise have their specific courses during pregnancy and lactation, are known to influence insulin sensitivity, and might also influence FA metabolism directly. To discuss each of these hormones individually is, however, beyond the scope of the current study.

Fatty acid sums and indices

To differentiate between mammary gland and hepatic enzymatic processes for *de novo* FA synthesis we applied the sum of FA that are, for the greater part, derived from DNL in the breast (ΣDNL-breast, sum of 6:0, 8:0, 10:0, 12:0, 14:0 and 14:1ω5) and the sum of FA that may at least partially derive from DNL in the liver (ΣDNL-liver, sum of 16:0, 16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9). The activities of hepatic DNL, SCD, D5D (Δ5-desaturase), D6D (Δ6-desaturase) were studied from their product/EFA ratios such as the DNL-activity indices (16:0/18:2ω6, 18:0/18:2ω6, 16:1ω7/18:2ω6, 18:1ω7/18:2ω6 and 18:1ω9/18:2ω6), their product/substrate ratios such as the SCD-activity indices (16:1ω7/16:0, 18:1ω7/16:0, 18:1ω9/18:0), the D5D activity index (AA/20:3ω6) and the D6D activity indices (20:3ω6/18:2ω6, DHA/EPA, 22:5ω6/AA).[45,47,65-70]

Statistics

Statistical analyses were performed with SPSS version 16.0.1 (SPSS Inc, Chicago, IL). Between-group differences were analyzed with student *t*-test or Mann Whitney U-test at $p<0.05$. Corrections were made for type-1 errors according to Bonferroni.

RESULTS

The fat content, MCSAFA, LCSAFA, MUFA, PUFA, LCP, DHA and AA compositions and the FA-sums and ratios that were calculated for preterm and term colostrum, transitional and mature breast milks are depicted in **Table 1**. The directions of the significant changes are presented to visualize consistencies.

Table 1. Fatty acid composition and fatty acid indices for enzymatic activities of preterm and term colostrum, transitional and mature milks

	milk fatty acid composition in g/100g FA and g/g FA						***Direction of change***				
	Preterm milk			*Term milk*			**PT vs. T**			**M vs. C**	
Milk (n)	*Colostrum (14)*	*Transitional (23)*	*Mature (13)*	*Colostrum (30)*	*Transitional (19)*	*Mature (34)*	**C**	***T***	***M***	**PT**	**T**
Fat % (g/L)	22.2 (15.6-41.9)	28.2 (17.7-58.8)	26.5 (18.3-67.4)	21.4 (13.7-32.8)	30.2 18.9-45.1)	30.6 (22.4-70.1)***					
MCSAFA (≤14:0)	16.9 (6.90-29.8)§§	30.9 (14.8-36.4)	33.7 (17.4-44.6)***	7.92 (4.71-25.4)§§	29.4 (10.8-39.9)	29.0 (10.2-29.7)***	↑			↑	↑
LCSAFA (≥16:0)	32.3 (27.3-36.8)	27.6 (22.3-35.5)	25.5 (23.7-30.6)***	32.8 (27.0-39.9)	26.6 (22.5-30.0)	24.3 (19.2-31.9)***				↓	↓
MUFA	32.5 (24.8-35.1)§§§	25.4 (17.7-41.0)	22.6 (16.6-34.3)**	40.0 (19.9-46.5)§§§	24.2 (18.4-45.7)	26.5 (14.3-35.3)***	↓			↓	↓
DHA (22:6ω3)	1.11 (0.38-2.03)§	0.85 (0.19-1.62)§	0.75 (0.12-2.12)	0.81 (0.32-1.43)§	0.48 (0.28-1.18)§	0.53 (0.24-1.83)**	↑	↑			↓
AA (20:4ω6)	0.93 (0.58-1.85)	0.71 (0.54-1.08)	0.69 (0.40-1.04)§.*	1.08 (0.64-1.94)	0.67 (0.37-0.99)	0.55 (0.38-0.85)§.***			↑	↓	↓
PUFA	20.2 (12.5-27.4)	16.7 (9.04-24.7)	16.3 (13.3-23.3)	19.2 (12.9-27.4)	16.7 (11.4-26.0)	19.7 (9.24-26.9)					
LCP	4.39 (3.33-7.98)§§.***	3.29 (2.09-5.02)	3.17 (2.38-5.53)	4.37 (3.20-6.72)	2.89 (1.79-4.26)	2.61 (1.83-4.65)§§.***			↑	↓	↓
ΣDNL (breast)	16.9 (6.92-29.8)§§	30.9 (14.8-36.4)	33.7 (17.4-44.6)***	7.92 (4.70-25.4)§§	29.4 (10.8-39.9)	29.0 (18.2-51.9)***	↑			↑	↑
ΣDNL (liver)	57.6 (49.4-66.8)§§§	49.3 (40.3-62.8)	47.9 (38.3-55.7)***	66.9 (45.5-71.9)§§§	47.3 (42.7-67.8)	48.6 (35.7-61.7)***	↓			↓	↓
DNL (16:0/18:2ω6)	1.91 (1.01-2.99)	1.75 (0.98-4.20)	1.73 (1.00-2.58)	1.99 (1.00-3.15)	1.58 (0.89-2.59)	1.24 (0.57-3.77)**					↓
DNL (18:0/18:2ω6)	0.33 (0.19-0.59)	0.33 (0.17-0.81)	0.34 (0.16-0.47)	0.40 (0.18-0.64)	0.32 (0.17-0.56)	0.27 (0.17-0.72)**					↓
DNL (16:1ω7/18:2ω6)	0.18 (0.06-0.40)§	0.18 (0.06-0.40)	0.13 (0.08-0.31)	0.26 (0.05-0.83)§	0.15 (0.06-0.55)	0.11 (0.01-0.48)***	↓				↓
DNL (18:1ω7/18:2ω6)	0.16 (0.07-0.34)§	0.14 (0.06-1.74)	0.12 (0.05-0.22)§§.*	0.21 (0.06-0.62)§	0.12 (0.05-0.34)	0.08 (0.03-0.34)§§.***	↓		↑	↓	↓
DNL (18:1ω9/18:2ω6)	1.69 (1.05-2.76)§§	1.59 (0.76-4.10)	1.46 (0.78-2.39)*	2.17 (0.72-3.85)§§	1.68 (0.95-3.39)	1.45 (0.93-3.34)***	↓			↓	↓
SCD (16:1ω7/16:0)	0.10 (0.05-0.14)§	0.10 (0.05-0.18)	0.09 (0.04-0.12)	0.14 (0.04-0.28)§	0.09 (0.04-0.25)	0.08 (0.02-0.22)***	↓				↓
SCD (18:1ω7/16:0)	0.10 (0.07-0.13)§	0.08 (0.06-0.99)	0.08 (0.05-0.10)*	0.11 (0.06-0.20)§	0.07 (0.04-0.16)	0.06 (0.04-0.11)***	↓			↓	↓
SCD (18:1ω9/18:0)	4.89 (3.99-6.60)§	4.84 (3.71-6.13)	4.20 (3.22-5.58)§§.*	5.70 (3.61-7.44)§	5.24 (3.63-7.46)	5.31 (2.87-6.89)§§	↓		↓	↓	
FADS1 (20:4ω6/20:3ω6)	1.34 (0.76-2.12)§§	1.33 (0.78-2.19)	1.13 (0.41-1.99)	1.82 (1.14-2.40)§§	1.21 (0.67-2.58)	0.99 (0.64-1.89)***	↓				↓
FADS2 (20:3ω6/18:2ω6)	0.055 (0.03-0.08)	0.047 (0.02-0.07)	0.051 (0.03-0.06)§	0.044 (0.02-0.10)	0.043 (0.03-0.10)	0.036 (0.02-0.08)§.*			↑		↓
FADS2 (22:6ω3/20:5ω3)	12.4 (8.41-52.0)	10.1 (5.44-43.0)	9.89 (4.82-38.0)§	11.8 (3.67-47.0)	9.60 (5.79-45.0)	6.35 (3.75-20.0)§.***			↑		↓
FADS2 (22:5ω6/20:4ω6)	0.17 (0.09-0.21)§§	0.15 (0.09-0.22)	0.14 (0.08-0.29)	0.12 (0.08-0.20)§§	0.14 (0.08-0.28)	0.14 (0.07-0.25)	↑				

Legend: see next page

Legend Table 1.
Data are medians (ranges) in g/100 g FA and g/g fatty acids.
PT, preterm (28-36 wks gestation); T, term (37-42 wks gestation)
C, colostrum (2-5 days); T, transitional milk (6-15 days); M, mature (16-56 days)
Significanct differences between preterm and term milk are indicated by §, $p<0.05$, §§, $p<0.01$, §§§ $p<0.001$
Significant differences between colostrum and mature milk ar indicated by *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$
MCSAFA, medium-chain saturated fatty acids (≤14:0); LCSAFA, long-chain saturated-FA (≥16:0); MUFA, monounsaturated-FA; PUFA, polyunsaturated-FA; LCP, long chain polyunsaturated-FA.
DNL, de novo lipogenesis; ΣDNL (breast), sum of 6:0, 8:0, 10:0, 12:0, 14:0 and 14:1ω5; ΣDNL (liver), sum of 16:0, 16:1ω7, 18:1ω7, 20:1ω7, 18:1ω9, 20:1ω9 and 22:1ω9
SCD, stearoyl-CoA desaturase (delta9-desaturase); FADS1/2, fatty acid desaturase enzymes 1 and 2 (delta5 and delta-6 desaturase)
↑/↓ indicates a higher/lower content in preterm vs. term and mature vs. colostrum milk

Differences from colostrum to mature milk

Fat. The median milk fat content increased from 22.2 g/dL in preterm colostrum (mean 3.5 days postdelivery) to 26.5 g/dL in preterm mature milk (24.5 days), and from 21.2 g/dL (3.1 days) in term colostrum to 30.7 g/dL in term mature milk (27.3 days), although the former increase was non-significant.

SAFA, MUFA and DNL-indices. Independent of prematurity, milk ΣDNL-breast and MCSAFA increased consistently, while LCSAFA decreased. ΣDNL-liver and MUFA also decreased. The trend for all DNL- and SCD-activity indices was a highly consistent decrease with advancing lactation. However, these decreases were only consistently significant (for both term and preterm) for two DNL-activity indices (18:1ω7/18:2ω6 and 18:1ω9/18:2ω6) and one SCD-activity index (18:1ω7/16:0). Only term milk showed an additional decrease in three other hepatic DNL activity indices (16:0/18:2ω6, 18:0/18:2ω6 and 16:1ω7/18:2ω6) and one SCD activity index (16:1ω7/16:0), while only preterm milk showed a decrease in the 18:1ω9/18:2ω6 activity index.

PUFA. We noted consistent decreases of AA, while only term milk showed a decrease in DHA (22:6ω3), the D5D-activity index (20:4ω6/20:3ω6) and two D6D activity indices (20:3ω6/18:2ω6 and 22:6ω3/20:5ω3).

Differences between preterm and term milk

Fat. There were no significant differences between the fat contents of preterm and term counterparts milks.

SAFA, MUFA and DNL-indices. Preterm colostrum milk had higher ΣDNL(breast) and MCSAFA (≤14C-atoms), but showed no difference with respect to LCSAFA. In contrast to MCSAFA, preterm colostrum milk exhibited lower ΣDNL-liver and MUFA as compared to term colostrum. The indices for hepatic DNL (16:1ω7/18:2ω6, 18:1ω7/18:2ω6, 18:1ω9/18:2ω6, but not 16:0/18:2ω6 or 18:0/18:2ω6) and SCD activity (16:1ω7/16:0, 18:1ω7/18:0 and 18:1ω9/18:0) were lower in preterm compared to term colostrum milk. All differences between preterm and term milk SAFA, MUFA and FA-indices, as observed in colostrum, disappeared with advancing lactation, with the exception of the DNL activity

index 18:1ω7/18:2ω6 that became higher in preterm mature milk and that of the SCD activity index 18:1ω9/18:0 that was still higher in term compared to preterm mature milk.

PUFA and FA-desaturase-indices. Preterm milk contained higher DHA in colostrum and transitional milk, and higher AA in mature milk compared to corresponding term milk. The index for activity of D5D was lower in preterm compared to term colostrum milk, while the D6D activity index was inconsistently higher (only for 22:5ω6/20:4ω6). In mature milk, two D6D activity indices (20:3ω6/18:2ω6 and 22:6ω3/20:5ω3) were higher in preterm compared to term milk.

Milk fatty acid interrelationships.

Selected relations between milk FA were studied in more detail. We found a strong inverse relation between MCSAFA and MUFA in all milk samples of preterm and term delivering women ($R^2 = 0.83$, $p<0.001$). MUFA correlated inversely with PUFA in colostrum and transitional milks (**Figures 1A** and **1B**, respectively), but positively in term milk (**Figure 1C**), while MCSAFA correlated inversely with PUFA in mature milk (**Figure 1F**).

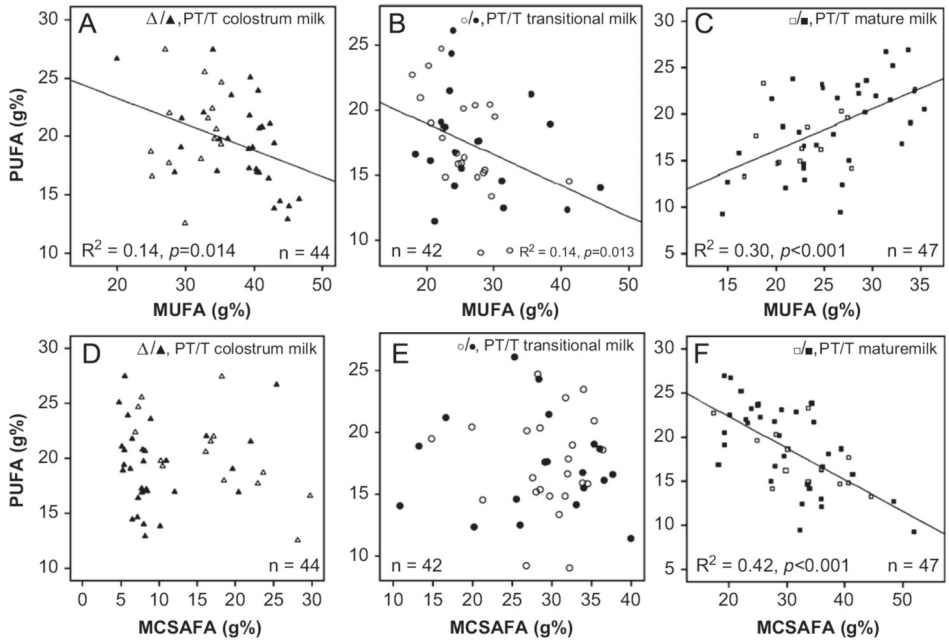

Figure 1. The relations between MUFA (panels A,B,C) and MCFA (panels D,E,F) on the one hand and PUFA on the other in colostrum (panels A,D), transitional milk (panels B,E) and mature milk (panels C,F) after preterm and term delivery. Data are in g/100g fatty acids (g%). MCFA, medium chain fatty acid (i.e. ≤14C-atoms); MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; Colostrum, 2-5 days; Transitional milk, 6-15 days; Mature milk,16-56 days; preterm delivery, 28-36 weeks; term delivery, 37-42 weeks. R2, correlation coefficient; p, significance of the correlation.

DISCUSSION

Milk from these African women showed much higher MCSAFA contents, higher LCP contents and lower MUFA contents compared to Western women (Table 1). These findings confirm the frequent consumption of freshwater fish in this African group[53] and reaffirm the higher MCSAFA and lower MUFA contents of African compared to Western milks, as previously observed by us[11] and others.[169,170] These differences have been attributed to the higher carbohydrate intakes of African women, but have, as yet, not been suggested to relate also to the hormonal levels around parturition.

Our results clearly indicate the increase of MCSAFA, and decreases of both MUFA and LCP with advancing lactation (Table 1). These results compare well with the data reported by others.[137,160-163] Part of these changes have been explained by the increase of the milk fat content[154,159,160] with advancing lactation. Secondly, compared to term milk, preterm milk contained higher MCSAFA in colostrum, higher DHA in colostrum and transitional milks, and lower MUFA in colostrum. Higher DHA in preterm milk has been reported before[150-155] and corresponding data of others also showed higher MCSAFA,[149,151,154-157] and lower MUFA, notably 18:1ω9[151,154-156,158] in early (≤10 days) preterm compared to term milk. Despite the high degree of consistency in the reported data, they remain largely unexplained. However, the importance of LCP, especially for the preterm infant,[171,172] has been emphasized and it has also been suggested that preterm infants might benefit from the slightly higher LCP levels in preterm milk.[149,150,157,171]

Total fat

The current lipid contents of preterm and term milk may not be comparable with the literature, since the present derive from the total FA contents of single breast milk samples taken during a feeding and thus not after emptying of the breast. However, given the reproducibility of the employed collection method, the current similarity between the lipid contents of preterm and term milks are of importance in this particular study, since they indicate that the observed FA differences between preterm and term milks are unlikely to be explained by differences in total fat contents.

Saturated-, ω7- and ω9-fatty acids

We used the milk MUFA, the sum of MUFA and 16:0 [abbreviated ΣDNL-liver] and the DNL- and SCD-activity indices as proxies for maternal insulin sensitivity and hepatic DNL synthesis. Justification comes from the work of Francois et al.[134] who showed that short term high intakes of 18:1ω9 and 16:0 have no influence on their milk contents, suggesting that these FA derive predominantly from a combination of hepatic DNL and long term dietary habits (i.e. stores). Adipose tissue lipolytic activity and hepatic DNL activity are known to be dependent on hormonal levels (e.g. insulin), glucose availability and LCPω3 status.[46,48,61,64] Since our data are in line with the literature and because the long term dietary habits of the investigated women were highly constant with little intra-individual and inter-individual variations due to the limited possibilities to change their dietary habits (e.g. LCPω3 intakes), we assume that the changes of milk MUFA, ΣDNL-liver and the DNL- and SCD-

activity indices are strongly dependent on corresponding changes in insulin sensitivity and glucose availability (or other hormonal changes around parturition), but not on dietary changes.

The reduced insulin resistance of late gestation leads not only to (compensatory) elevated insulin levels, postprandial glucose levels and adipose tissue lipolytic activity (**Figure 2**; see E) and thereby circulating FFA,[56] but also to insulin- and glucose- stimulated hepatic DNL (Figure 2; see A),[61] which, together with diminishing adipose tissue LPL activity (Figure 2; see D), results in increasing circulating levels of VLDL (Figure 2; see B).[56] Of further notice, it has been shown that parturition and subsequent suckling, irrespective of length of gestation, trigger the synthesis of MCSAFA in the mammary gland[136-138] (Figure 2). The observed higher MCSAFA content of preterm, compared to term colostrum, renders a higher MCSAFA synthetic activity after term delivery, unlikely. Glucose transport into the lactating mammary alveolar cell is considered insensitive to insulin,[129] with the exception of certain conditions, such as starvation. Furthermore, mammary gland LPL-activity is increased 1-2 days before parturition[139] and continues to increase during lactation[138-141] (Figure 2; see C), while the LPL-mediated FA uptake into adipose tissue becomes reduced by the insulin resistance of late pregnancy[140,173] (Figure 2; see D).

Consequently, in term milk, compared with preterm milk, one might expect both higher MCSAFA (due to the elevated postprandial glucose levels) and higher LCSAFA and MUFA (due to higher adipose tissue FFA release, higher hepatic DNL activity and thus higher contents in the VLDL-TG, and higher mammary gland LPL-activity). We indeed observed higher MUFA (Table 1), but lower MCSAFA and no difference in LCSAFA (Table 1), in term compared to preterm colostrum, suggesting that mothers who deliver preterm have higher mammary MCSAFA-synthesis, despite lower postprandial plasma glucose levels. However, since parturition and not length of gestation triggers MCSAFA synthesis and because glucose uptake in the mammary gland is mostly insulin insensitive, the observed differences, i.e. the higher MCSAFA and lower MUFA in preterm compared to term colostrum (Table 1), are not likely to be explained by higher MCSAFA synthesis *per se*. The strong inverse relation between MCSAFA and MUFA in milk lipids, rather suggests competition (Figure 2; see 2) between *de novo* synthesized FA in the mammary gland (with MCSAFA as a proxy) and those taken up by the mammary gland either as FFA or via LPL (with MUFA as a proxy). It seems (Figure 2), consequently, that when the mammary gland influx of notably MUFA is low (such as after preterm delivery), higher amounts of MCSAFA will become incorporated into milk lipids, and *vice versa*. Secondly, the higher MCSAFA content of preterm colostrum seems unhampered by the concurrent lower postprandial glucose levels that come along with preterm delivery, suggesting that the circulating glucose level is not the principal driving force of mammary gland MCSAFA synthesis

In line with the above, the present preterm milk FA composition indeed suggests a lower maternal adipose tissue FFA release and a lower hepatic DNL activity after preterm compared to term delivery. This became mainly noticeable from the lower hepatic DNL end-products in preterm colostrum, such as 16:1ω7, 18:1ω7, 18:1ω9 and 20:1ω9 (MUFA), but not by lower 16:0 and 18:0 (i.e.

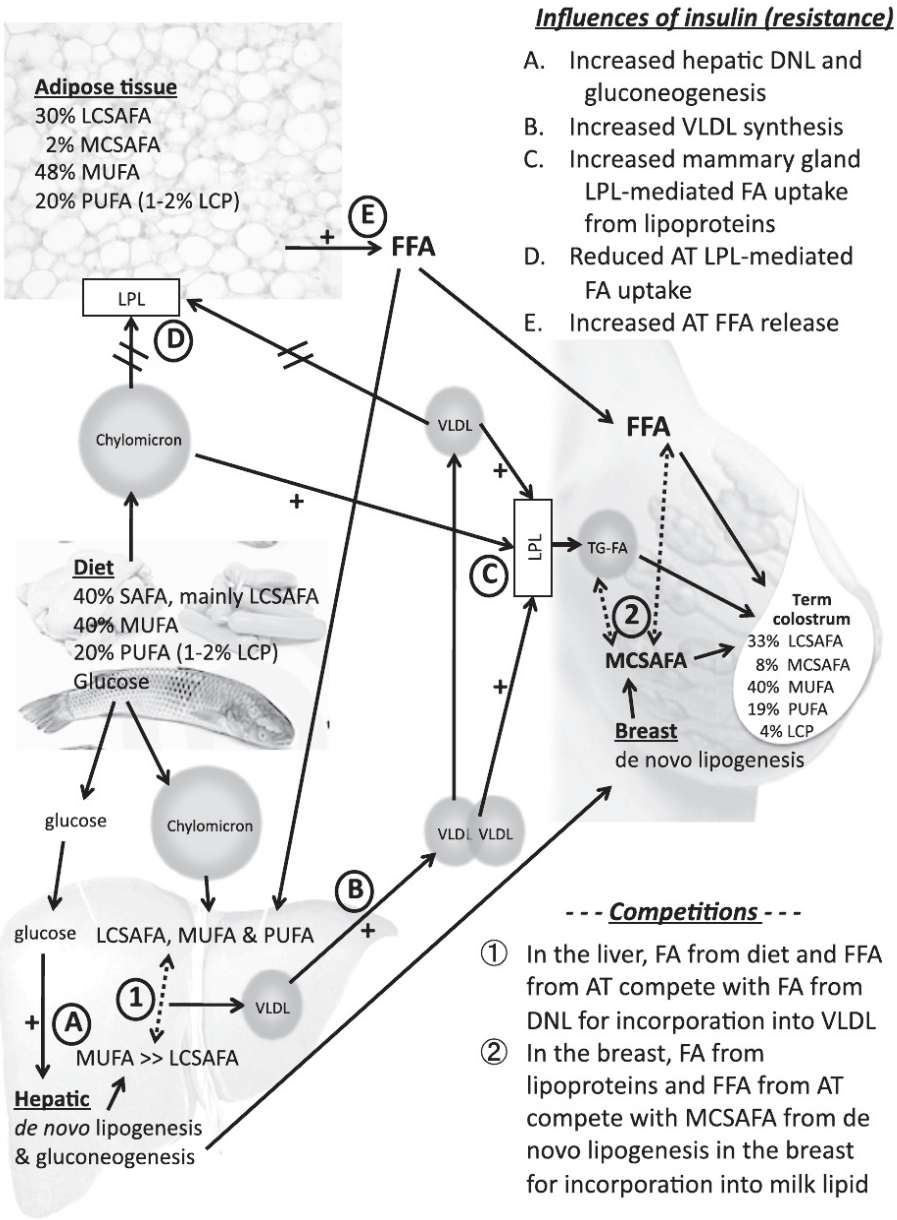

Figure 2. Proposed effects of insulin sensitivity on the fatty acid composition of preterm and term colostrum and with advancing lactation.

Breast milk FA (right side) derive from adipose tissue, the diet and liver (all on the left side), and *de novo* FA synthesis in the mammary gland (right side). Dietary FA in chylomicron-TG are transported to the tissues where they are taken up via an LPL mediated process. Chylomicron remnants are taken up by the liver and their FA, together with FFA from adipose tissue (process E) and *de novo* synthesized FA (from glucose; process A), are incorporated into TG, that are subsequently secreted as VLDL (process B). FA from VLDL-FA are taken up by the mammary gland via LPL (process C), where they, together with FFA (from adipose tissue; process

E) and mammary gland *de novo* synthesized FA (i.e. MCSAFA; from glucose), are incorporated into milk TG. In the liver, FA from dietary origin (LCSAFA, MUFA and PUFA) compete with *de novo* synthesized FA (LCSAFA, but notably MUFA) for incorporation into VLDL-TG (Process 1). In the mammary gland, FA from VLDL (LCSAFA, MUFA, PUFA) and FFA from adipose tissue (LCSAFA, MUFA, PUFA) compete with *de novo* synthesized FA in the mammary gland (MCSAFA) for incorporation into milk TG (Process 2). The state of reduced insulin sensitivity/compensatory hyperinsulinemia of the third trimester promotes processes A, B, C and E, while process D is downregulated. Term birth is characterized by a more pronounced state of reduced insulin sensitivity/compensatory hyperinsulinemia , which implies higher stimulation of processes A, B, C and E. Term birth is therefore associated with higher hepatic *de novo* FA synthesis (with MUFA as a marker), higher *de novo* synthesized FA in VLDL and better competition of the hepatic *de novo* synthesized FA with *de novo* synthesized FA in the mammary gland (with MCSAFA as marker), which becomes noticeable by higher MUFA and lower MCSAFA in term colostrum compared with preterm colostrum. Rapid normalization of the reduced insulin sensitivity/compensatory hyperinsulinemia after delivery causes a rapidly diminishing *de novo* FA in the liver and diminishing of MUFA in VLDL, which ultimately causes in both preterm and term milks a decrease of MUFA that is compensated for by an increase of *de novo* synthesized MCSAFA in the mammary gland
Single headed arrows indicate transport of the nutrient from one place to another; double headed arrows indicate competition. The positive (+), negative (-) or absent (no) signs indicate the influence of insulin on the magnitude of the process. The average FA compositions of adipose tissue, the Western diet and of term colostrum milk are indicated. Data are in g/100 g (g%) fatty acids (FA). Abbreviations: LCSAFA, long chain saturated fatty acids (≥16:0); LCP, long chain polyunsaturated fatty acids (≥20C, ≥3 double bonds); LPL, lipoprotein lipase; MCSAFA, medium chain saturated fatty acids (≤14:0); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids (≥2 double bonds), TG, triglycerides; VLDL, very low density lipoprotein.

LCSAFA), suggesting that hepatic DNL and hepatic FA-metabolism (of dietary and adipose tissue derived FA) is aimed at the synthesis of MUFA rather than LCSAFA. This suggestion is supported by the lower 16:1ω7/18:2ω6; 18:1ω7/18:2ω6 and 18:1ω9/18:2ω6 ratios (as proxies for hepatic DNL activity) in preterm milk, while this was not observed for the 16:0/18:2ω6 and 18:0/18:2ω6 ratios. Mechanistically, the observed absence of differences between preterm and term colostrum in 16:0, 18:0, and the DNL-indices 16:0/18:2ω6 and 18:0/18:2ω6 in our study and those of others[149-156,158] might be explained by the efficiency of hepatic SCD and Elovl-6 to convert 16:0 to 16:1ω7, 16:1ω7 to 18:1ω7, 16:0 to 18:0, and 18:0 to 18:1ω9, and is probably driven by the compensatory hyperinsulinemia of late gestation.[61,63] The important influence of insulin in these conversions might additionally be supported by the decreases in 16:0/18:2ω6, 18:0, 18:0/18:2ω6, 16:1ω7 and 16:1ω7/18:2ω6 in term milks with the duration of lactation (Table 1). These decreases coincide with the well known restoration of insulin sensitivity/compensatory hyperinsulinemia and, as is the case in our data, might be more readily observed in the milk of term delivering women because of their more pronounced postpartum changes in insulin sensitivity, compared with preterm delivering counterparts.

Our suggestion of a causal relationship between a higher milk MUFA content and lower insulin sensitivity also becomes supported by the higher MUFA in mature milk of Westernized overweight urban women compared to normal weight rural South African women[169] and also by the higher MUFA in milk of many Western women compared to non-Western counterparts.[129,170] A lower insulin

sensitivity in Western pregnancies (Figure 2), stimulates mammary gland LPL-activity (C), FFA release from adipose tissue (E) and hepatic DNL [i.e. LCSAFA and MUFA synthesis (A); followed by VLDL synthesis and secretion (B)]. The subsequent higher FA uptake from VLDL in the mammary gland competes successfully with mammary gland DNL (i.e. MCSAFA synthesis; Figure 2, see 2). Furthermore, the currently studied women had a much higher DHA status compared to most European women, while DHA is known as a strong suppressor of hepatic DNL.[64] In other words, the currently observed much higher milk MCSAFA, and thus lower MUFA, in developing countries is usually explained by the higher carbohydrate intakes in these countries, but might at least in part also be due to the inhibitory effect of a higher DHA status on hepatic DNL-activity.[45,64] Other support for a relation between the milk FA composition and insulin sensitivity comes from the higher 18:1ω9 content in milk of diabetic women.[174] The high proportions of MCSAFA (39-53 g%), in the context of a somewhat lower milk fat content, in a patient with type I hyperlipoproteinemia, in which there is a genetically-determined deficit of LPL-activity,[175] also supports a competition between LPL-mediated FA uptake with *de novo* MCSAFA synthesis in the breast.

The postpartum restoration of insulin sensitivity after both preterm and term deliveries became reflected by decreases in the milk MUFA with concomitant increases in MCSAFA (Table 1). The rapidity[55] by which insulin sensitivity is restored after delivery (about 3 days) is likely to be the cause of the rapid vanishing of all FA differences, resulting into no differences in LCSAFA, MCSAFA and MUFA between preterm and term transitional and mature milks.

In summary, the data on the milk MCSAFA (a proxy for DNL in the mammary gland) and MUFA (a proxy for adipose tissue FFA release, and hepatic DNL and VLDL synthesis) show that these distinct sources compete for incorporation into milk lipids (Figure 2). The competition is likely to be governed by insulin sensitivity. Mothers who deliver at term are less insulin sensitive than those who deliver preterm. We suggest that in term delivering mothers, this difference causes higher FFA release from adipose tissue, higher DNL and VLDL-synthesis in the liver and a higher mammary gland LPL-activity (Figure 2). Term colostrum is consequently higher in MUFA and thus lower in MCSAFA. The restoring insulin sensitivity after delivery causes a decrease of MUFA and an increase of MCSAFA in both preterm and term milks with the duration of lactation, resulting in the similarity of the transitional and mature milk FA compositions. This change is more pronounced in term milk, since mothers who deliver at term experience more pronounced changes of the insulin sensitivity than counterparts who deliver preterm. Taken together, the MCSAFA and MUFA differences between preterm and term milk and their changes with the duration of lactation suggest that mammary gland DNL is secondary to LPL-mediated and/or direct uptake of (F)FA from the maternal circulation, which might in turn be driven by the insulin insensitivity of late pregnancy. The favored uptake of FA from the circulation might not only aim at the provision of an important energy source, but also at the postdelivery transfer of important tissue building blocks, such as 18:1ω9, PUFA and LCP.

PUFA

In accordance with our data,[89] inconsistent increases of ALA and LA[137,176,177] have been observed with advancing lactation, while no differences are observed between preterm and term milks.[150-152,155,156,158] Since mammary gland LPL-activity is stimulated by insulin,[16] suggesting less LPL-activity after preterm delivery, one may expect lower MUFA and PUFA in preterm colostrum and a compensatory increase of milk MCSAFA. Although lower MUFA and higher MCSAFA were indeed observed (see above), lower PUFA was not (Table 1). The inverse correlation between MUFA and PUFA in colostrum and transitional milks even suggest higher PUFA, with lower MUFA in preterm milks (Figures 1A and 1B). Thus, the absence of any difference in PUFA between preterm and term milks, except for AA and DHA (see below), suggests additional influences that tend to keep milk PUFA constant. The equal or higher PUFA in preterm colostrum can be explained (Figure 2) by a combination of a lower circulating VLDL[56] in conjunction with a higher VLDL-PUFA content immediately after preterm delivery. A higher VLDL-PUFA after preterm delivery might result from the lower amount of notably MUFA (from maternal stores and *de novo* MUFA synthesis) that is available for competition with PUFA for incorporation into VLDL (Figure 2; see 1). The postnatal interaction in the liver between FA derived from DNL and PUFA was characterized by the changes in their relations in colostrum, transitional and mature milks, respectively (Figure 1, A-C). These showed that in the colostrum-to-mature milk sequence of restoring insulin sensitivity, the initially weak competition (Figure 1 A and B) between MUFA (as a proxy for hepatic DNL-FA) and PUFA changed to collaboration (Figure 1 C). Meanwhile, there was no correlation between MCSAFA and PUFA in colostrum and transitional milks (Figures 1D-E), but this changed into competition in mature milk (Figure 1F). We suggest that the hepatic DNL-FA, which are more abundantly synthesized after term birth, initially compete successfully for incorporation into VLDL (Figure 2, see 1) at the expense of those from the diet and stores, but that this competition vanishes as a function of insulin sensitivity restoration and turns into the joint competition of MUFA and PUFA with MCSAFA for incorporation into milk lipids (Figure 1F; Figure 2, see 2). As such, it seems that, by virtue of the higher insulin sensitivity after preterm birth, the relatively higher PUFA content in VLDL is able to compensate for the lower circulating VLDL concentration and the lower mammary gland LPL-activity, with the net result that preterm and term milks do not harbor much difference in their PUFA contents.

In a mathematical model (not shown), we evaluated the effects of different contributions of MUFA and LCSAFA from adipose tissue lipolysis (increasing FFA; i.e. higher LCSAFA, MUFA and PUFA) and hepatic DNL [increasing MUFA and LCSAFA; and increasing VLDL, i.e. increasing LCSAFA, MUFA and PUFA)] on the resulting MUFA, LCSAFA and PUFA levels in milk. Calculations were performed at fixed milk MCSAFA levels (10, 20 and 30 g%) and a fixed relative contribution of adipose tissue PUFA (70%) and dietary PUFA (30%) to the total milk PUFA.[78] The outcome confirmed that i) milk MUFA decrease secondary to lower FA contributions from adipose tissue and hepatic DNL (i.e. with increasing insulin sensitivity), while ii) milk LCSAFA and iii) milk PUFA are quite resistant to changes in these contributions.

LCP

In contrast to the similarity of ALA, LA and PUFA[150-152,155,156,158] between preterm and term milks and their apparent constancy, or small increases, with advancing lactation (Table 1), many authors have reported higher LCP in premature milk,[150-153,156,157] while there is also a highly consistent LCP decrease with advancing stage of lactation.[161,162] In this study, we found higher DHA in premature colostrum and transitional milks and higher AA in premature mature milk (Table 1), compared to term milk. We also found that LCP, DHA (only for term) and AA decreased with advancing lactation (Table1). These well-documented observations have, to our knowledge, not as yet been explained in terms of preterm-term differences in insulin sensitivity or its postdelivery restoration.

Fat output increases with advancing lactation,[154,159,160] together with the size of the fat globules,[145,147,148] which causes a decrease of the PL/TG ratio.[148] Since LCP have a preference for incorporation into PL,[137,143] this decrease in the PL/TG ratio coincides with a decrease of the contribution of LCP to the total milk FA content. The mechanism for the increase in fat globule size is unknown but may be related to the maturation of the mammary gland.[129,148] We suggest that the physiological restoration of insulin sensitivity shortly after delivery might influence the decrease of the milk LCP percentage with advancing lactation. The higher relative LCP content of colostrum compared to mature milk might relate to the higher LCP levels in the livers of pregnant compared to non-pregnant animals,[178] while these levels decrease rapidly after the initiation of lactation. The high hepatic LCP content is likely to result from the high insulin levels at the pregnancy-end,[54] since insulin not only induces hepatic DNL (see above) but also augments the activities of the enzymes involved in EFA-desaturation; i.e. D5D and D6D.[46,48] Hence, the rapid postpartum restoration of insulin sensitivity [48] may reduce hepatic LCP synthetic activity and thereby decrease the contribution of LCP to the total milk FA contents with advancing lactation. Our data indeed suggest postpartum decreases of some of the proxies of D5D and D6D activities, which, because of the more pronounced changes in insulin sensitivity of women delivering at term, may notably have become reflected in term milk (Table 1).

Predicting the net effect of a higher maternal insulin level, such as encountered after term delivery, on the milk LCP content is difficult, since the state of reduced insulin sensitivity/ compensatory hyperinsulinemia of late pregnancy promotes not only the release of FFA from adipose issue and hepatic DNL (mainly SAFA and MUFA), but also LCP synthesis by augmention of D5D- and D6D-activities. It seems that the 'LCP-diluting' contributions that come from the LCP-poor adipose tissue FA and DNL-FA (Figure 2) increase with advancing gestation, since preterm colostrum and transitional milk exhibited higher DHA compared to term milk, but many other mechanisms may also be involved.

Our data argue against a more pronounced maternal LCP depletion[79] as an explanation for the frequently observed[150] lower DHA and AA contents in term compared to preterm colostrum in *Western* infants. The currently investigated African women have lifetime high fish intakes and are therefore unlikely to become DHA depleted between preterm delivery and term age.[166]

Nevertheless, their preterm colostrum also harbored higher DHA than term colostrum. Interestingly, since no differences were observed between the average fat globule sizes nor the PL contents of preterm and term milks,[142-145] the currently observed LCP differences, i.e. *higher* DHA, but non-significantly *lower* AA, between term and preterm colostrum indicate that besides insulin, other factors might be involved.

The suggested relation between insulin levels and D5D and D6D activities in pregnancy, might especially be important for the understanding of the LCP needs of preterms, since these become largely devoid of any LCP, maternally synthesized LCP included, that are normally transferred across the placenta from the time of delivery to term age. The ensuing "LCP gap" becomes even greater if the infant subsequently receives infant formula without LCP. The understanding of maternal LCP synthesis in pregnancy might also be important to diabetic pregnancies, where the stimulatory effects of the high insulin levels on the D5D- and D6D-activities might become offset by the D5D and D6D-inhibitory effects of the concomitantly increased glucose levels.[46,48] Moreover, those LCP that are synthesized may under these conditions become even more 'diluted' by the more abundantly *de novo* synthesized FA from the liver.[15,50] Both the insulin resistance and the high glucose levels may under these conditions promote lipogenesis via LXR and SREBP-1c, and ChREBP, respectively.[61]

Summarizing the observations on PUFA and LCP, we suggest that the absence of differences between the LA, ALA and PUFA contents of premature and term milks, and the absence of changes in these FA with duration of lactation may result from several opposing mechanisms that keep these levels constant (Figure 2). Mechanisms that tend to increase milk PUFA are the higher FFA release from adipose tissue stores, the higher plasma VLDL concentrations (more VLDL synthesis), and the higher mammary gland LPL activity (more FA uptake from lipoproteins) that comes together with the lower insulin sensitivity/higher insulin of i) women who deliver at term and ii) early lactation. A milk PUFA decreasing mechanism is the dilution of PUFA from the diet and stores by DNL FA in the liver, that comes together with the same conditions.

With regard to LCP we suggest that the decreasing milk LCP content and decreasing PL/TG ratio in both premature and term milks with advancing lactation may relate to the postnatally decreasing D5D and D6D-activities, secondary to the restoring insulin sensitivity. The higher DHA in premature, compared to term, colostrum and transitional milk in the current study population is unlikely to relate to depletion of maternal stores. LCP are more likely to be dependent on the same mechanisms as outlined for PUFA (i.e. adipose tissue FFA release, serum VLDL, mammary gland LPL-activity and hepatic DNL) with in addition their insulin-promoted synthesis from precursors via augmentation of D5D- and D6D-activities.

CONCLUSIONS, LIMITATIONS AND IMPLICATIONS

We propose that the FA-differences between preterm and term milks are for a considerable part caused by the higher maternal insulin sensitivity after preterm birth, compared with term birth. The restoring insulin sensitivity after delivery may be an important factor in the FA-differences

between colostrum, transitional and mature milks. Our interpretations should be viewed upon as hypotheses that need confirmation from future measurements of a combination of hormones and FA, and notably by measurements of enzymatic activities and *in vivo* fluxes with e.g. stable isotopes in pregnancy and lactation.

The current study may add to the understanding of the mechanisms that cause differences in the body compositions of preterm and term infants and may thereby ultimately provide insight into the "gaps" that have to be filled by nutritional means. For instance, our data suggest that the preterm infant is devoid of several weeks of transplacental transfer of insulin-induced synthesis of MUFA deriving from maternal hepatic-DNL and from LCP that are likely to derive in part from insulin-induced maternal hepatic D5D and D6D-activities. MUFA, notably oleic acid, might be important for myelination.[94] Meanwhile, premature milk contains lower MUFA, while its somewhat higher LCP vanishes rapidly with advancing lactation and will not be able to fill the growing "LCP gap". Conversely, premature infants are at present precociously exposed to the high levels of LA and MCSAFA in human milk and formula, while both of these are low *in utero*. The possible benefits of a high LCP intake by preterm infants have been demonstrated in trials,[171,172,179] but no studies have been performed with higher milk MUFA and lower milk LA and MCSAFA contents. Any lowering of the LA and MCSAFA intakes must on the other hand be weighed against the building of the skin's water barrier by LA[180-185] and the ease by which MCSAFA carrying TG are hydrolyzed in the gastrointestinal tract.[186] To arrive at a balanced catch-up growth of preterm infants it is necessary to know which 'windows of opportunity' are still open and which are definitely closed. The mimicking to some extent of the intrauterine conditions, characterized by high mother-to-infant fluxes of both MUFA and LCP, and a low LA accretion, might in premature infants be important to the growth of lean body mass and neurodevelopment, including myelination.

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CHAPTER 8

Saturated fat, carbohydrates and cardiovascular disease

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ABSTRACT

The dietary intake of saturated fatty acids (SAFA) is associated with a modest increase in serum total cholesterol, but not with cardiovascular disease (CVD). Replacing dietary SAFA with carbohydrates (CHO), notably those with high glycemic index, is associated with an increase in CVD risk in observational cohorts, while replacing SAFA with poly-unsaturated fatty acids (PUFA) is associated with reduced CVD risk. However, replacing a combination of SAFA and *trans*-fatty acids with n-6 PUFA (notably linoleic acid) in controlled trials showed no indication of benefit and a signal toward increased coronary heart disease risk, suggesting that n-3 PUFA may be responsible for the protective association between total PUFA and CVD. High CHO intakes stimulate hepatic SAFA synthesis and conservation of dietary SAFA. Hepatic *de novo* lipogenesis from CHO is also stimulated during eucaloric dietary substitution of SAFA by CHO with high glycemic index in normo-insulinemic subjects and during hypocaloric high-CHO/low-fat diets in subjects with the metabolic syndrome. The accumulation of SAFA stimulates chronic systemic low-grade inflammation through its mimicking of bacterial lipopolysaccharides and/or the induction of other pro-inflammatory stimuli. The resulting systemic low-grade inflammation promotes insulin resistance, reallocation of energy-rich substrates and atherogenic dyslipidemia that concertedly give rise to increased CVD risk. We conclude that avoidance of SAFA accumulation by reducing the intake of CHO with high glycemic index, is more effective in the prevention of CVD, than reducing SAFA intake *per se*.

INTRODUCTION

In 2003 in the Netherlands, fat comprised about 34% of total energy intake (en%), and was, after carbohydrates (CHO), the main energy source. Grains, grain products and non-alcoholic beverages are the most important source of dietary CHO in the Netherlands (**Table 1**). Saturated (SAFA), mono-unsaturated and poly-unsaturated fatty acids (PUFA) each constitute approximately 12.9, 10.8 and 7.1 en% of the total fat intake. Milk, milk products and meat are the main sources of dietary SAFA (Table 1) in the Netherlands,[1,2] as well as in the United States.[3] Fatty acids are pleiotropic nutrients with important functions in the human body in addition to serving as substrates for energy production. Fatty acids are essential components of the phospholipids in all cell membranes, act as carriers of the fat-soluble vitamins A, D, E and K, and include the essential n-3 and n-6 PUFA, alpha-linolenic and linoleic acid, respectively.

The 'diet-heart hypothesis,' also named the diet-heart paradigm, is based on the association between serum cholesterol and dietary SAFA with the risk of cardiovascular disease (CVD) that was found in the Seven Countries Study by Ancel Keys,[4] and the relationship between dietary SAFA and serum cholesterol that was demonstrated in short[5] and long-term feeding trials.[6,7] However, recent evidence from RCTs and observational studies have provided little support for the diet-heart paradigm and the causality of the association between dietary SAFA and CVD outcomes is increasingly questioned.[8-11]

In this paper we discuss the current scientific data on the effects of dietary SAFA, their controversies and the potential underlying (patho)physiological mechanisms for the role of SAFA in CVD.

Lipoproteins, cholesterol, SAFA and CVD

A high serum total cholesterol, and especially LDL-cholesterol, is associated with an increased risk of CVD, whereas a high HDL-cholesterol has a protective association.[12] The serum total cholesterol/HDL-cholesterol ratio is the consensus risk factor for the estimation of coronary heart disease risk. The reduction of this ratio by 1 point is classically associated with a coronary heart disease risk reduction of 52%.[13] The metabolic syndrome, also called the "insulin resistance syndrome", which is characterized by obesity, impaired glucose homeostasis, hypertension and atherogenic dyslipidemia ("the deadly quartet"), is a major risk factor for CVD.[14] Similarly, atherogenic dyslipidemia,[15] which is characterized by elevated triglycerides, "small dense LDL" particles and reduced HDL-cholesterol ("deadly lipid triad"),[16] is a major risk factor for CVD. These small dense LDL particles are susceptible to structural modifications by oxidation[17] and notably oxidized LDL particles effect atherosclerotic plaque formation[18] by promoting foam cell generation, endothelial dysfunction and local inflammation.

An increase in the consumption of SAFA by 1 energy percent (en%), raises serum total cholesterol by 0.052 mmol/l.[6] However, in the same study, the total cholesterol of subjects consuming 15 en% SAFA, ranged between 4 and 6 mmol/l, indicating that most variation in the serum total cholesterol

Table 1. Dietary intakes and the average contributions of different dietary resources in 2003 in a typical Dutch population[a,b]

	Energy	Protein	Fat	SAFA	MUFA	PUFA	*trans*	CHO	MS & DS	PS	Fibre
Total daily intake (kcal and g/day)	2328	81	90	33	28	19	3	277	144	133	19.3
Total daily intake (en%)		14.3	34.4	12.9	10.8	7.1		48.2	24.9	23.1	2.1
Potatoes and other tuberous organs (g%)	3.8	2.9	1.2	1.7	1.0	0.8	3.4	6.2	0.7	12.2	15.1
Vegetables (g%)	1.1	2.2	0.3	0.2	0.5	0	0	1.3	1.6	1.0	12.2
Legumes (g%)	0.1	0.3	0	0	0		0	0.2	0	0.4	1.1
Fruits[c] (g%)	3.9	2.6	3.7	1.9	4.9	6.1	0	4.8	8.2	1.1	12.3
Milk and milk products (g%)	14.5	25.1	18.1	30.8	13.0	2.5	19.8	9.9	17.8	1.3	1.9
Cheese	5.0	9.2	10.7	18.0	7.9	1.5	13.3	0.1	0.2	0	0
Grains and grain products[d] (g%)	23.0	20.8	10.8	6.7	9.2	17.2	25.4	34.0	5.2	65.2	43.0
Bread	16.4	16.9	5.8	3.6	4.1	11.3	3.8	25.3	4.1	48.3	35.9
Meat and meat products[e] (g%)	11.4	29.9	20.1	20.3	25.9	10.3	10.0	0.7	0.2	1.2	0.1
Fish and shellfish (g%)	0.5	2.1	0.6	0.4	0.8		0.2	0.1	0	0.2	0
Eggs (g%)	0.7	1.7	1.3	0.8	1.4		0.1	0	0	0	0
Fats[f] (g%)	6.4	0.1	18.8	14.5	16.5	33.5	13.1	0	0.1	0	0
Sugar and candy (g%)	7.7	1.7	5.2	6.3	5.5	3.6	3.3	11.7	20.6	2.1	3.6
Cookies, cake and biscuits (g%)	7.0	3.2	7.7	9.0	7.2	5.2	18.6	8.3	7.7	8.9	4.7
Non-alcoholic beverages (g%)	8.2	1.6	0.2	0.3	0.1	0	0.1	16.3	30.6	0.8	1.8
Alcoholic beverages (g%)	5.1	1.0	0.1	0.1	0.1	0	0.1	2.9	4.4	1.3	0
Sauces, seasonings, herbs and spices (g%)	4.0	0.9	9.4	4.4	12.3	16.3	0.7	1.5	2.1	0.9	0.8
Soups and bouillon (g%)	0.8	1.3	0.8	0.8	0.8		0.7	0.6	0.3	0.9	1.9
Miscellaneous (g%)	1.7	2.5	1.8	1.9	1.2		4.4	1.5	0.7	2.4	1.4
Total	100	100	100	100	96	96	100	100	100	100	100

[a]. data derived from the Dutch VCP reference population (n=750) of Dutch men (352) and women (398) between 19-30 years of age in 2003 (1.2).

[b]. SAFA. saturated fatty acids; MUFA. monounsaturated fatty acids; PUFA. polyunsaturated fatty acids; trans. trans-fatty acids; CHO. carbohydrates; MS. monosaccharides; DS. disaccharides; PS. polysaccharides

[c]. including seeds and nuts

[d]. including flour. bread. pasta. rice and cereals

[e].including poultry

[f]. including oils. butter. margarines and other frying fats

is not on account of the differences in SAFA intake *per se*.[6] Because of the supposed relationship between SAFA and CVD, it is nowadays recommended to replace dietary fat, and especially SAFA, by *cis*-unsaturated fatty acids.[19,20] Between 1987/1988 and 1997/1998 the intake of *cis*-unsaturated fatty acids, refined CHO and mono-and disaccharides in the Netherlands increased at the expense of fat, SAFA and *trans*-fatty acids.[19,20] In this review, we will evaluate the current consensus on the relationship between SAFA and CVD while the consequences of replacing SAFA by CHO, mono-unsaturates and PUFA will be examined with regard to their influence on atherogenic dyslipidemia.

SAFA and CVD

A recent meta-analysis of prospective cohort studies[21] showed that the intake of SAFA is not associated with an increased risk of coronary heart disease, stroke or those two combined (i.e. cardiovascular disease, CVD), before[21] or after[22] adjustment for serum total cholesterol. Additionally, the consumption of milk and milk products was not related to CVD in a meta-analysis of prospective cohort studies. Consumption of milk and milk products may even decrease CVD risk,[23] although this meta-analysis of prospective cohort studies was not supported by a recent prospective cohort study in the Netherlands.[24]

Replacing SAFA by CHO

Replacing 5 en% SAFA with 5 en% CHO, reduced serum total cholesterol by 0.18 mmol/l, LDL-cholesterol by 0.16 mmol/l and HDL-cholesterol by 0.05 mmol/l, increased the serum triglycerides by 0.11 mmol/l and had no effect on the total cholesterol/HDL-cholesterol ratio.[5] LDL-cholesterol reduction from isocaloric substitution of SAFA by CHO is accompanied by an increase in the amount of atherogenic small-dense LDL particles and a decrease in the less atherogenic large LDL particles[15] Because of the increase of triglycerides and the small dense LDL and no change in the total cholesterol/HDL-cholesterol ratio, replacing SAFA by CHO seems unfavorable with regard to CVD prevention.

A pooled analysis of 11 cohort studies showed that replacing 5 en% SAFA by CHO was associated with a slightly increased risk of coronary events (7%), but there was no difference in mortality[25] In practice, however, SAFA are often replaced by CHO, notably by CHO with a high glycemic index. A subsequent analysis by Jakobsen et al.[26] showed that replacement of 5 en% SAFA by CHO with a low glycemic index was associated with a non-significant reduction in CVD risk, while replacing SAFA by CHO with a high glycemic index was associated with a 33% increased risk of myocardial infarction[26]

Replacing SAFA by mono-unsaturated fatty acids

Replacing 5 en% SAFA by 5% mono-unsaturated fatty acids reduced total cholesterol by 0.21 mmol/l, LDL-cholesterol by 0.20 mmol/l, and HDL-cholesterol by 0.01 mmol/l, and increased the serum triglycerides by 0.01 mmol/l. The 0.145 reduction in the total cholesterol/HDL-cholesterol ratio is predicted to translate to a coronary heart disease risk reduction of 7.5%.[13] However, in the

recent pooled analysis of prospective observational cohorts by Jakobsen et al.,[26] the intake of mono-unsaturated fatty acids was associated with a 19% higher risk of CVD events, but not with cornary heart disease mortality. This outcome contrasts with the beneficial effects of the so called Mediterranean diet, which is typically high in mono-unsaturated fatty acids,[27] and the theoretical decrease of the total cholesterol/HDL-cholesterol ratio, when SAFA are replaced by mono-unsaturated fatty acids.[5] Consequently, it was recently concluded that there is insufficient evidence to advise the replacement of SAFA by mono-unsaturated fatty acids.[28]

Replacing SAFA by n-6 and n-3 PUFA

Replacing 5 en% SAFA by 5 en% PUFA decreased total cholesterol by 0.29 mmol/l, LDL-cholesterol by 0.26 mmol/l, HDL-cholesterol by 0.02 mmol/l, triglycerides by 0.03 mmol/l and the total cholesterol/HDL-cholesterol ratio by 0.175, which theoretically corresponds to a coronary heart disease risk reduction of 9.1%.[13] A pooled analysis of 11 cohort studies showed that replacement of 5 en% SAFA by (n-3 and n-6) PUFA was associated with a significant 13% reduction in coronary events and a 26% reduction in coronary heart disease mortality.[25] These results are consistent with a meta-analysis of RCTs,[29,30] which showed that replacing 5 en% SAFA by (n-3 and n-6) PUFA reduced coronary heart disease risk by 10%. These results have been interpreted as providing strong concordant evidence to support current recommendations to substitute the n-6 PUFA linoleic acid for SAFA, and were recently translated into an American Heart Association (AHA) advice,[31] to consume 'at least 5 to 10 % of energy as n-6 PUFA'. Importantly however, neither of these pooled analyses made a clear distinction between n-6 and n-3 PUFA species, and the Mozaffarian et al.[29] meta-analysis of RCTs did not consider the potential confounding role of *trans*-fatty acids. The n-3 PUFA[32] and *trans*-fatty acids[33] have been positively and negatively related to CVD development, respectively. If the distinction is made between interventions that selectively substituted the SAFA and *trans*-fatty acids by n-6 PUFA/linoleic acid, and those that substantially increased both n-3 and n-6 PUFAs, a whole different picture emerges.[34] Linoleic acid selective PUFA interventions produced no indication of benefit but rather a fairly consistent, but non-significant, signal toward *increased* risk of coronary heart disease and death. These potentially negative effects of n-6 PUFA acid may even have been underestimated, since PUFA also replaced *trans*-fatty acids. If SAFA is replaced by both n-3 and n-6 PUFA, a significant (22%) decreased coronary heart disease risk is found. However, this reduction may also be attributable, at least in part, to the reduced consumption of *trans*-fatty acids.[34]

Risk reduction in perspective

Besides the already mentioned large variability in the relationship between serum total cholesterol and SAFA intake,[6] there is the well known example of the African Maasai who have very high intakes of both cholesterol (500-2,000 mg/day) and SAFA from milk,[35,36] but exhibit remarkably low serum cholesterol levels[8,11,35,36] and although accompanied by extensive atherosclerosis, with lipid infiltration and fibrous changes, they have a very low incidence of cardiovascular events[8]

Secondly, comparison with other risk factors and the feasibility of a reduced SAFA consumption also require some attention. In 2003 the average SAFA intake in the Netherlands was estimated at 12.9% of total energy (en%). This intake should be lowered by 38%, i.e. to 7.9 en%, to achieve a 10% risk reduction in CVD.[30] The Dutch National Institute for Public Health and the Environment (RIVM) calculated that a 5% reduction in SAFA intake would reduce the annual incidence of CVD with 4,300 persons per year and CVD mortality with 1,000 people per year,[37] at an annual mortality from CVD of approximately 40,000/year in the Netherlands.[38] For comparison, the estimated mortality attributable to overweight, insufficient fruit and vegetable intake, and low fish intake are 6,900, 7,300, 4,500 persons per year, respectively.[37] A recent report from the UK predicted that an increase in the intake of fruits and vegetables from 279-356 g to 440 g/day would save as many lives as a reduction in the current SAFA consumption in the UK from >14 to 3 en% and a reduction in salt intake from >8.1 to 3.5 g/day.[39] Consequently, other risk factors seem much simpler to be addressed and their role seems at least comparable, if not more important, in the current high incidence of CVD. Recommendations to increase intakes of n-3 PUFA, fruit and vegetables and reduce sodium intakes,[30] to increase physical activity,[40] to reduce *trans*-fatty acid intakes[30] and reduce the intake of CHO with high glycemic index and glycemic load, such as notably found in soft drinks and candy (Table 1), seem more prudent candidates in the battle against CVD,[41] than a recommendation to reduce SAFA intakes by 10 en%, and also because SAFA are in daily practice mostly replaced by CHO with high glycemic index.[42]

The relation between inflammation and lipoprotein metabolism

The causal relationship between LDL-cholesterol *per se* and CVD[6] is still a matter of debate.[8-11] However, both oxidized and small dense LDL have been related to increased CVD risk.[17,18] Moreover, there is convincing evidence that the LDL-cholesterol reducing statins reduce CVD risk,[43-46] but statins have pleiotropic effects. Statins also have an anti-inflammatory effect and equally reduce CRP and the concentration of LDL-cholesterol.[43-47] This observation supports the endotoxin-lipoprotein hypothesis[48] stating that "chronic systemic low grade inflammation" connects LDL-cholesterol to CRP. It is becoming clear that changes in serum lipoproteins might be a response to a state of chronic inflammation *secondary* to our current lifestyle that in its turn is composed of many factors. Besides the influence of dietary changes, environmental changes such as stress, sleep deprivation and environmental pollution, including smoking, have also been related to chronic inflammation.[49] It was recently re-emphasized that these so called gene-environment interactions play important roles in de development of many, if not all, current diseases of civilization,[50,51] while a primary role of 'faulty' genes, seems grossly overestimated.[52,53]

Common metabolic disorders, such as obesity, type 2 diabetes and the metabolic syndrome are associated with low grade inflammation and elevations in acute phase proteins such as CRP.[54] It has become increasingly clear that insulin resistance develops secondary to systemic inflammation and the compensatory hyperinsulinemia aims primarily at balancing glucose homeostasis.[55,56] The

insulin resistant state, induced by pro-inflammatory cytokines, is indispensable for the reallocation of energy-rich substrates. Glucose is conserved for the metabolically highly active brain and for the activated immune system, which both rely on glucose metabolism for their energy supply.[55] Organs that would normally also use glucose become insulin resistant and use triglycerides and free fatty acids, distributed by the liver and adipose tissue, respectively, as energy supplies. At the same time, the lipoprotein profile might act to fight off inflammation and support the repair of tissue damage secondary to the inflammatory reaction.[57-66] This is executed via: 1) an increase in cholesterol-rich lipoproteïns (mainly LDL and VLDL), that have the ability to bind bacterial lipopolysaccharide (LPS) in proportion to their cholesterol content,[16,67,68] although the best determinant of the capacity of lipoprotein to bind LPS is a high phospholipid/cholesterol ratio (i.e. surface/volume ratio)[69] 2) the suppression of reverse cholesterol-transport via multiple pathways[66] (i.e. low HDL) 3) increased oxidation of LDL and VLDL, while HDL becomes proinflammatory[62] 4) increased cholesterol delivery to the immune system[62] and 5) the production of "small dense" LDL particles.[70] The latter become enriched in sphingolipids, are poorly cleared by the LDL-receptor, cross the endothelial barrier more effectively, bind to the vascular wall intima and are accumulated in macrophages because of their susceptibility to oxidative modification.[62] Taken together, the proatherogenic dyslipidemia of the metabolic syndrome is in support of the recovery from inflammation-induced damage.[48,55] However, when these changes in the lipoprotein profile last for prolonged periods of time, such as in the chronic low-grade inflammation of the metabolic syndrome,[14] they give rise to the development of atherosclerosis.[62,66]

It is increasingly clear that our current lifestyle includes many factors that 1) initiate and propagate inflammation, 2) give rise to an inadequate capacity to terminate inflammatory responses, and 3) lead to insufficient protection from the "collateral damage" caused by the chronic immune activation. One of these factors is our dietary SAFA intake, that can cause inflammation by their mimicking of a part of bacterial LPS[48] and/or by providing other pro-inflammatory stimuli.[67,68,71] Whether dietary SAFA cause inflammation depends on the accumulation of SAFA in the body and not on the dietary SAFA intakes *per se*. Accumulation of SAFA can also occur by the synthesis of SAFA from CHO via *de novo* fatty acid synthesis. This mainly occurs in the liver, which secretes these *de novo* synthesized fatty acids as VLDL.[16,72]

SAFA vs. carbohydrates, the metabolic syndrome and the immune system

The adverse effects of high SAFA intakes on lipid metabolism, are particularly noted when SAFA are combined with high CHO intakes. Under these conditions, dietary SAFA are preserved, while the surplus of the consumed CHO is converted to SAFA by hepatic *de novo* fatty acid synthesis. Although the conservation of SAFA during excessive intake of CHO with a high glycemic index is well known,[73-76] the synthesis of SAFA from (surplus) CHO may not have received sufficient attention. Contrary to widespread belief, *de novo* fatty acid synthesis is not restricted to hyper-caloric conditions or to excessive intakes of CHO, but also depends on the type of ingested CHO.

A low-fat eucaloric diet with a high sugar/starch ratio stimulated *de novo* fatty acid synthesis and increased serum triglycerides in normal weight individuals.[72] When subjects with the metabolic syndrome, i.e. with pre-existing insulin resistance, were fed either a hypocaloric low-CHO/high-fat diet with high SAFA content or a hypocaloric high-CHO/low-fat diet with low SAFA content, the low-CHO/high-SAFA diet resulted in *lower* SAFA levels in plasma lipids compared to the high-CHO/low-SAFA diet.[75,76] Finally, in subjects with the hepatic manifestation of the metabolic syndrome, i.e. non-alcoholic fatty liver disease, 26% of the fatty acids in the liver-triglycerides and 23% of the fatty acids in VLDL- triglycerides derive from *de novo* fatty acid synthesis in the liver.[77] Importantly, as much as 25-30% of Western adults are suffering from non-alcoholic fatty liver disease[78] in which the hepatic synthesis of fat, including SAFA, has become independent of the metabolic state, i.e. is independent of the feeding-fasting-cycle.[77] Taken together, SAFA accumulate: 1) under eucaloric conditions in normal weight subjects who consume a CHO-rich diet with high glycemic index and 2) under hypocaloric conditions in subjects with the metabolic syndrome and non-alcoholic fatty liver disease who consume CHO-rich diets. Thus CHO, particularly those with a high glycemic index, and pre-existing insulin resistance are confounding factors in the discussion on the relation between CVD and dietary SAFA. This observation underscores the importance of a renewed discussion about the possible dangers of dietary SAFA.

CONCLUSIONS

The total body of evidence suggests that attention should be shifted from the harmful effects of dietary SAFA *per se*, to the prevention of the accumulation of SAFA in body lipids. This shift would emphasize the importance of reducing dietary CHO, especially CHO with high glycemic index, rather than reducing dietary SAFA. The chronic interaction of SAFA with our immune system elicits so called "chronic systemic low grade inflammation", which underlies the metabolic changes referred to as the (atherogenic) dyslipidemia of the metabolic syndrome or the lipidemia of sepsis. The ultimate goal of the ensuing insulin resistance is the re-allocation of energy-rich substrates, such as glucose, to the immune system while the change in our lipoprotein profile aims at the limitation of the inflammatory responses and the repair of the resulting tissue damage. Dietary SAFA belong to the many false triggers of inflammation that result from the conflict between our slowly adapting genome and our rapidly changing lifestyle, but among these many factors they are not the most important. A reduction in the consumption of CHO with a high glycemic index, *trans*-fatty acids and linoleic acid, and an increased consumption of fish, vegetables and fruit, and a reduction of inactivity, sleep deprivation and chronic stress seem more realistic approaches to fight the current pandemic of cardiovascular disease resulting from chronic systemic low grade inflammation.

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CHAPTER 9

Summary

ABSTRACT

Introduction. Darwin described two forces in evolution: 'natural selection' and 'the conditions of existence', of which, in his mind, the latter was the most important. Our genetic material (genome) adapts very slowly to the rapidly changing conditions of existence. Evolutionary medicine teaches us that typical diseases of civilization, such as cardiovascular disease, diabetes, certain cancers and degenerative brain diseases are caused by a 'mismatch' between the manmade rapidly changing conditions of existence and our ancient genome. For prevention and treatment, it is necessary to acknowledge the changes in our conditions of existence that have occurred since the agricultural and the industrial revolutions and to obtain detailed insight into these changes.

Materials and Methods. We performed a multi-disciplinary literature study of the conditions of existence of our distant ancestors who lived in East-Africa. With these data we reconstructed the composition of various East-African diets in the Paleolithic era. We also collected milk, red blood cells, umbilical cord vessels and adipose tissue of traditionally living East-African peoples for the determination of their respective fatty acid compositions.

Results. Our literature study demonstrated that our hunting and gathering ancestors lived in the land-water ecosystem, and therefore derived at least part of their diet from this niche. Compared to our current Western diet, they had lower intakes of carbohydrates and linoleic acid, but higher intakes of protein and the typical fish oil fatty acids EPA and DHA. Our field research in East-Africa supported a high intake of fish oil fatty acids from the local land-water ecosystems.

Conclusions. Differences between our current Western diet and the diet of our Paleolithic ancestors contribute strongly to the many years that Western populations live with chronic diseases. The search for answers to the question of how to improve this situation is however hampered by the present paradigm that prescribes how studies of the health effects of our diet and lifestyle should be performed. It appears simple and that is exactly what it is: for healthy aging we need to return to the Paleolithic conditions of existence, as translated into the culture of the 21st century.

1.1 Introduction to Evolutionary Medicine and aim of this thesis

The Dutch Nobel prizewinner Nikolaas Tinbergen first described the important differences between proximate (direct) and ultimate (evolutionary) causes for animal behavior[1]. His observations were extended to disease occurrence by the pioneers of 'Evolutionary Medicine', i.e. Randolph M. Nesse and George C. Williams[2,3]. Proximate explanations explain *how* things (e.g. a disease) happen, while evolutionary explanations explain *why* things (e.g. diseases) occur. This difference is of great importance, but seems poorly appreciated in health care. In medical practice, proximate explanations deal mostly with treatment (health care), while evolutionary explanations are useful for both primary and secondary prevention (public health and health care, respectively). By their (predominantly drug) treatment, physicians in modern (Western) medicine focus mostly on the proximate (mechanistic) causes of disease[4], with little attention for the secondary prevention lessons that can be learned from evolutionary medicine. The latter emphasizes the importance of ultimate explanations of diseases, since these, rather than the treatment of their proximate causes, provide targets for the reduction of the burden of chronic disease, the suffering thereof and their associated costs. Evidence Based Medicine (EBM) has greatly contributed to the current gap between proximate and evolutionary medicine, since it focuses preferably on the outcomes of interventions with single (chemical) components in patients with preexistent pathologies, rather than on holistic approaches to increase and/or preserve health in healthy subjects, such as advocated in Evolutionary Medicine. Moreover, such holistic approaches are popularly often dismissed as 'alternative' medicine. Meanwhile, a surprisingly high percentage of about 70% of the Dutch population is considered to 'believe' in Evolution while 10% is 'not sure'[5].

Evolutionary medicine acknowledges that many of the present day diseases of civilization, including cardiovascular disease, diabetes mellitus, certain cancers, certain neuropsychiatric diseases and osteoporosis, result from conflicts between our rapidly changing environment and our only slowly adapting genome, which basically still resides in the Paleolithic era. This conflict can only be solved by gaining knowledge of our ancient environment and our behavior in this environment (lifestyle), since man-orchestrated engineering of our genome to make us adapt to the 21st century environment is no realistic option. This rapidly changing environment includes insufficient physical activity, lack of sleep, altered bacterial flora and exposure to environmental pollution and chronic stress. Besides these important changes, our diet is an important environmental factor. Our diet has experienced tremendous changes in the recent past, but it might, among the various lifestyle factors, be one of the easiest to modify back to what it originally was. For this, reconstruction of our diet prior to the onset of the agricultural and industrial revolutions is indicated, but there is still much uncertainty about the ecological niche in which we became to what we currently are. However, what scientist do agree on is that the major part of human evolution occurred in (East) Africa.

This thesis describes studies that have been initiated with the Evolutionary Medicine concept in mind. For many of these we went 'back-to-Africa'. Our goal is the treatment and notably prevention of typically Western diseases by looking back into the past, when according to the rules of evolution

our genome became adapted to our ancient environment. Our contribution aims ultimately at the modification of our current lifestyle and notably certain aspects of our contemporary diet with the goal to reduce the burden of degenerative diseases by the promotion of healthy ageing according to the lessons of Darwin, i.e. 'adaptation to the conditions of existence'.

1.2 Human origins and the early human niche

In **Chapter 1** we update the evidence for healthy ageing among our early ancestors and in the late and present hunter-gatherer societies. We criticize the long reigning paradigm that our ancestors evolved on the hot and arid savannah and argue that they rather found an ecological niche in the proximity of water. Evidence from various disciplines is discussed, including the study of Paleoenvironments, comparative anatomy, biogeochemistry (which relates isotope abundances in fossils to dietary habits of the proprietor), archeology, anthropology, (patho)physiology and epidemiology, and the study of Paleolithic diets that have been reconstructed from naturally available foods. We also review the limited number of studies that have thus far investigated the health effects of the putative Paleolithic diets. We conclude that there is ample evidence that longevity was not uncommon among hunter-gatherers. While ageing provided an direct evolutionary advantage with regard to the quantity of offspring, the presence of these older individuals also provided a subsequent evolutionary advantage to their offspring. These observations suggest that there has been ample opportunity for the evolution of healthy ageing. We also conclude that our ancestors evolved in a land-water ecosystem and extracted a substantial part from their diets from these environments. Rather than rejecting this possibility by a lack of evidence, we propose that the default assumption should be that *hominins*[*], living in coastal ecosystems with catchable aquatic resources, consumed the aquatic resources found in their immediate vicinity.

2.1 The early human diet

In **Chapter 2** we reconstructed multiple Paleolithic diets to estimate the ranges of nutrient intakes upon which humans evolved. We composed a database of, predominantly East-African, plant and animal foods (including both meat and fish) to model multiple Paleolithic diets at known hunter-gatherer plant/animal food intake ratios [range 70/30 to 30/70 energy% (en%)]. We applied two pathophysiological constraints to the models. The first was that protein intakes needed to be below 35 en%; while the second constraint was that the intakes of linoleic acid (18:2ω6; LA) needed to be above 1.0 en%. In the past, Boyd Eaton, Loren Cordain and Michael Crawford, who co-authored the study, had addressed similar questions, but had more or less concentrated on either a terrestrial diet or an aquatic diet, or restricted their reconstruction to a fixed plant/animal intake ratio. Additionally, much emphasis had been put on the observation that our ancestors acquired the use of stone tools

*A human or a human ancestor, including all of the *Homo* species (*Homo sapiens, H. ergaster, H. Rudolfensis,* etc.), all of the Australopithecines (*Australopithicus africanus, A. boisei*, etc.) and other ancient forms like *Paranthropus* and *Ardipithecus*.

about 3.4-2.4 million years ago and developed the skills to get access to bone marrow and brain from hunted or scavenged animals. Consequently, there had been ongoing discussion whether long-chain polyunsaturated (LCP; ≥3 double bonds, ≥20 C-atoms) fatty acids (FA) derived either from the aquatic food-chain (i.e. from fish) or might also have been acquired from the terrestrial food-chain, through the consumption of animal brains after the cracking of their skulls with stone tools. Regarding the typically opportunistic way of life ascribed to hominins, we investigated the most plausible scenario in which *hominins* consumed a mixed diet from both terrestrial and aquatic resources, with a wide range of dietary intakes from both plant and animal food resources. Therefore, we investigated selective and non-selective savannah, savannah/aquatic and aquatic hunter-gatherer/scavenger foraging-strategies. Within the scavenger foraging-strategy, we additionally varied the amount of organ meats consumed (brain, liver, bone marrow and adipose tissue) within reasonable limits.

Despite the wide range of dietary possibilities, the outcomes resulted in a remarkably uniform dietary intake pattern. The macronutrient composition of the presumed Paleolithic diet averaged from 25-29 en% protein (overall range 8-35 en%); 39-40 en% carbohydrates (CHO; 19-48 en%) and 30-39 en% (20-72 en%) fat. Thus, the various Paleolithic diets provided moderate-to-high protein and fat intakes and moderate CHO intakes, when compared to the current international and national (Dutch) intakes and recommendations and were quite similar to, for example, the South Beach diet (for other examples: see Table 6 in Chapter 1). The reconstructed diets were high in saturated FA (SAFA) (11.4-12.0 en%) and moderate-to-high in monounsaturated FA (MUFA) (5.6-18.5 en%) and polyunsaturated FA (PUFA, ≥2 double bonds, ≥18 C-atoms) (8.6-15.2 en%). PUFA intakes were notably explained by consisted α-linolenic (18:3ω3; ALA) (3.7-4.7 en%), LCPω3 (2.26-17.0 g/day) and LCPω6 (2.54-8.84 g/day) intakes, whereas LA intakes were remarkably low (2.3-3.6 en%), compared with current Western intakes and recommendations. Compared to Western diets, Paleolithic diets contained consistently higher protein and LCP, and lower LA. These are likely to contribute to the known beneficial effects of Paleolithic-like diets. For instance, hypercaloric CHO intakes have been implicated in dyslipidemia, while increased protein intakes have been related to satiety and satiation. Disparities between Paleolithic, contemporary and recommended intakes might be important factors underlying the etiology of common Western diseases. Data on Paleolithic diets and lifestyle, rather than the investigation of single nutrients, might be useful for the rational design of clinical trials.

Milk fatty acid compositions in traditional East African populations

3.1 High in AA and DHA

Chapter 3.1 questions the validity of current recommendations for the arachidonic acid (20:4ω6; AA) and docosahexaenoic acid (22:6ω3; DHA) contents of human milk, in view of the profound dietary changes in the past 100 years. Despite these changes, current recommendations are based on the average breast milk compositions of Western women. In search of the milk AA and DHA

contents of our African ancestors we initiated an investigation of a Tanzanian population living on the shores of Lake Kitangiri,(see Figure 1, Scope) where local corn oil-fried fish proved to be the major lipid source, while corn porridge and local fruits and vegetables constitute their staple foods. We collected milk samples from 29 lactating women and conducted FA analysis of these milks and of the fish species available on the local markets. These women exhibited some dietary differences compared to the presumed Paleolithic diet (i.e. higher CHO and LA acid intakes). A multivariate analysis showed that the milk medium-chain SAFA (MCSAFA; which derive from endogenous FA synthesis from glucose in the mammary gland) and LA contents did not affect the milk AA and/or DHA contents. The high milk AA (0.70 mol%) and DHA (0.75 mol%) and low AA/DHA ratio (0.91 mol/mol) traced to the consumption of freshwater fish with both high AA and DHA contents and a high AA/eicosapenaenic acid (20:5ω3; EPA) ratio. We concluded that the milk AA, DHA and the EPA (0.17 mol%) contents of these women might be used to optimize infant formulas and the milk of Western women. Beneficial neurodevelopmental outcomes after supplementation of pregnant women or infants with LCP, however, proved difficult to attest in controlled trials thus far, possibly because of the plasticity of the brain and the subtle effects involved. However, the Barker hypothesis predicts that the ultimate results of inadequate supply of certain nutrients during intrauterine development and similarly during the period of exclusive breastfeeding will affect health after the reproductive period, that is increase the prevalence of chronic degenerative disease at the end of the life cycle.

3.2 Low in LA, high in MCSAFA and LCP

In a subsequent study, described in **Chapter 3.2**, we investigated the milk FA composition of several Tanzanian tribes (for locations see Figure 1, Scope). Our primary objective was to differentiate between women consuming salt water and freshwater fish, and to compare the outcomes with those from women with very low fish intakes due to cultural beliefs or the simple absence of fish in the near environment. We collected milk samples from 20 fish-consuming women living in the island of Chole, located in the Indian Ocean, 30 women living in the Ukerewe island in Lake Victoria, 30 women living at Matema beach at the north-western shore of Lake Malawi, and 35 women from the shores of Lake Kitangiri. For non-fish eaters we collected milk from 28 traditional hunting and gathering Hadzabe women, from 27 traditionally living pastoral Maasai women, from 9 neighboring Sonjo women, and from 18 Iraqw women who live on the foothills of Mount Hanang. The milk of the women with high fish intakes showed high mean levels of LCP, ranging from 0.50-0.80 g% AA and from 0.53-1.79 g% DHA. The mean level of 1.79 g% proved the highest milk DHA content reported in the literature so far through analysis using capillary GC columns. Women living in the islands of Chole and Ukerewe showed remarkably low mean levels of LA in their milks (range 4.23-5.20 g%). High levels of MCSAFA, notably 12:0 and 14:0, compensated for the low levels of LA. The low levels of LA were explained by the low intake of vegetable oils. Chole women had high levels of both 12:0 (20.17 g%) and 14:0 (21.19 g%), while Ukerewe women had moderate levels of 12:0 (12.28 g%) and high levels of 14:0 (20.26 g%), which were explained by their regular intakes of 12:0 and 14:0

from coconuts and their high CHO intakes, respectively. The AA/DHA ratios in the fish-eating groups related strongly to the AA/DHA ratios in the available fish. Importantly, the milk of a substantial part of these fish eating populations surpassed the upper level for the 12:0 and 14:0 contents of infant formulas, while LA levels did not meet the recommendation. Conversely, the milk of a substantial part of the inland Tanzanian tribes did not meet the minimum intakes for both AA and DHA such as issued by several Western nutritional boards.

3.3 Western recommendations criticized

Despite the publication of Chapters 3.1 and 3.2, the authors of a subsequent meta-analysis[6] of the world wide human milk DHA and AA contents concluded that the average values of 0.32 g% DHA and 0.47 g% AA resulting from their search should serve as a guide for infant feeding. In **Chapter 3.3** we discuss the consequences of their point of view and we propose that the evidence-based recommendations issued by various health organizations, i.e. to consume up to 3 servings of fish per week, should be translated into a recommendation for the breast milk LCP content and hence for the daily LCP intakes of an infant. In other words, a guide for infant feeding should not derive from the milk composition of women who typically do not fulfill the well-founded recommendation to consume up to 3 servings of fish a week.

3.4 Differences between preterm and term milk in East-Africa

Studies in Western subjects show a decrease in the milk AA and DHA contents with the duration of lactation, which is often explained by depletion of the maternal stores. Similarly, the higher levels of DHA and AA in preterm compared to term milk are explained by the depletion of maternal LCP stores that comes along with advancing gestation. In **Chapter 3.4** we discuss whether the same effect is observed in women with lifelong high LCP intakes from freshwater fish, since these high intakes might be expected to prevent the depletion of maternal LCP stores. For this purpose we collected preterm and term colostrum, transitional and mature milk samples from women living in Sengerema, at the southern shore of Lake Victoria (Figure 1, Scope). We collected 30 colostrum, 19 transitional and 34 mature breast milk samples from women who delivered at term. These numbers were 14, 23 and 13, respectively, for women who delivered prematurely. In our study population, both DHA and AA decreased from colostrum to mature milk, i.e. both after preterm and after term delivery. The observed median DHA (1.11-0.75 g%) and AA (0.93-0.69 g%) levels in preterm milk proved the highest among the reported contents of the worldwide preterm milks so far. The outcome substantiates our previous statement in **Chapter 3.3** that most of the reported human milk compositions derive from studies with Western women with relatively low fish intakes. Consistent with the literature, DHA proved higher in preterm compared to term colostrum, suggesting that other influences than the depletion of maternal stores might have caused the observed difference in this particular population with high DHA intakes from freshwater fish. We suggest that the postpartum increase in MCSAFA, and higher MCSAFA in preterm, compared to

term colostrum, as well as the decreasing MUFA with advancing lactation, and the lower MUFA in preterm compared to term colostrum, might derive from differences in maternal insulin sensitivity. Insulin sensitivity might ultimately govern the relative contributions of *de novo* synthesized FA from the liver and those from the mammary gland. An elaboration on the influence of insulin sensitivity on FA metabolism can be found in **Chapter 6**.

Erythrocyte fatty acid compositions of traditional East-African populations

4.1.1 Three African tribes with negligible, Western-like, and high fish intakes

In **Chapter 4** we describe the courses of erythrocyte (red blood cell; RBC) FA during the perinatal period and across the life cycle. In addition, we investigated whether biomagnification, i.e. the higher LCP in infant compared to maternal plasma phospholipid (PL) and RBC (in g/100 g FA), also occurred in women with very high fish intakes. Similar to the decreasing milk fat DHA content with the duration of lactation, the maternal plasma PL- and RBC-DHA contents are known to decrease during pregnancy and subsequent lactation in Western women. Similarly, the maternal AA status decreases during pregnancy, but increases again in late pregnancy or early lactation. We investigated whether these observed changes were similar in Tanzanian women with low, intermediate or high fish intakes. For this, we focused on three diverse populations who all live in Tanzania, East-Africa (see Scope: Figure 1; locations 2, 9 and 10).

The Maasai are pastoralist people from Nilo-Hamitic descent. They inhabit the Maasai Plains that range from the Kenyan border far into the South of Tanzania. Their original life is centered around their cattle and their original diet was composed of milk and meat. Governmental pressure has pursued many of these people to abandon their nomadic lifestyle and settle, which also resulted in an increase of the incorporation of cereals, mainly corn porridge, into their diets. However, fish are still not eaten due to their cultural belief that it is a snake-like creature and therefore not edible. Maasai are remarkably fit and apparently free of cardiovascular disease. The Maasai were included for the near absence of preformed DHA from fish in their diet. The second group lives at the base of the Pare Mountains in the east of Tanzania. The Pare Mountains are quite fertile and provide the inhabitant Pare and Sambaa tribes with abundant corn, rice, wheat, fruit and vegetables, while both meat and fish are affordable for most of the villagers. Cattle is locally abundant and additionally brought in by Maasai from the Maasai Plains in the west who trade their cattle for a wide range of items available in the Pare villages. Fish is obtained from nearby lakes, of which the closest is located about 30 kilometers away. Their dietary fish intakes were estimated at 2-3 times/week, but the consumed fish rarely exceed the maximum of 15 cm in size. The final study population inhabits the shores of Lake Victoria near Sengerema and consists of a variety of local, mostly Bantu, tribes. Since we performed our project in a local governmental hospital, visiting patients were all from the local lower and middle class, since upper class people attend the private hospitals in these countries. Consequently, most of these families obtained the majority of their dietary protein from fish, since at a lakeshore; these are much cheaper than meat. Local fish range from 15 cm to over 1

meter (average 30 cm) in size and from ground dwelling catfish, prehistoric lungfish, tilapia and the recently introduced Nile Perch, to the decimated endemic cichlids of the Lake, locally known as furu, that however, have begun to recover in multitude again, although most have lost their beautiful colors. Importantly, the available sardine species, locally known as dagaa, are considered as a vegetable and are actually consumed as being butter to bread in Western societies. We estimated that the average fish intake ranged from 4-5 times/week in this population.

4.1.2 Intrauterine bioattenuation and infant DHA equilibrium: 6 g% RBC-DHA

In **Chapter 4.1**, we investigated whether biomagnification persists at lifelong high maternal fish intakes. Secondly, we investigated whether the consistently observed decreases in the infant and maternal DHA status after delivery were also observed in the population with high fish intakes. We collected blood samples and analyzed their FA contents in 14 women during the first trimester, 103 women during the second trimester and 88 women during the third trimester of pregnancy. We also analyzed blood samples from 63 mother-infant pairs at delivery and 103 mother-infant pairs after 3 months of exclusive lactation. We found that at delivery and after 3 months of breastfeeding the infants of all three tribes had remarkably similar RBC-AA, which were unrelated to maternal RBC-AA. RBC-AA decreased in infants after delivery, but meanwhile increased in maternal RBC. A second finding was that at delivery the Maasai and Pare women had lower RBC-DHA compared to their infants, but that the RBC-DHA of the Sengerema women was higher than that of their infants. From these data, we calculated that transplacental biomagnification (i.e. a higher infant compared to maternal DHA status at delivery) disappears when the mother and infant reach an (equilibrated) status of 5.6 g% DHA in their respective RBC. In addition, our data revealed that at higher maternal RBC-DHA, infants had lower RBC-DHA compared to their mothers. We coined this lower infant compared to the maternal RBC-DHA content at high maternal fish intakes 'bioattenuation,' as opposed to the well-known process of 'biomagnification' resulting in a higher infant compared to maternal DHA status (**Figure 1**). After delivery, however, the maternal RBC-DHA content decreased consistently, suggesting higher infant DHA demands during lactation, compared to pregnancy. This decrease in maternal RBC-DHA resulted in a lower maternal, compared to infant, RBC-DHA content after 3 months lactation in all tribes. Importantly, however, the concomitant changes in infant RBC-DHA related to the maternal status; showing a decrease in the Maasai infants, no change in the Pare infants and an increase in Sengerema infants. From these courses of infant RBC-DHA after delivery, we estimated that the postpartum infant RBC-DHA content remains constant from an initial status of 6 g%, which corresponds to a maternal RBC-DHA content of 6 g% throughout pregnancy. We concluded that a uniformly high infant AA status at delivery might indicate the importance of a certain intrauterine infant AA-status. Secondly, we propose that biomagnification reflects a low maternal DHA-status and that bioattenuation may prevent intrauterine competition of DHA with AA. Finally, we concluded that a maternal RBC-DHA content of 6 g% during pregnancy predicts maternal-fetal equilibrium at delivery and postnatal infant RBC-DHA equilibrium. However,

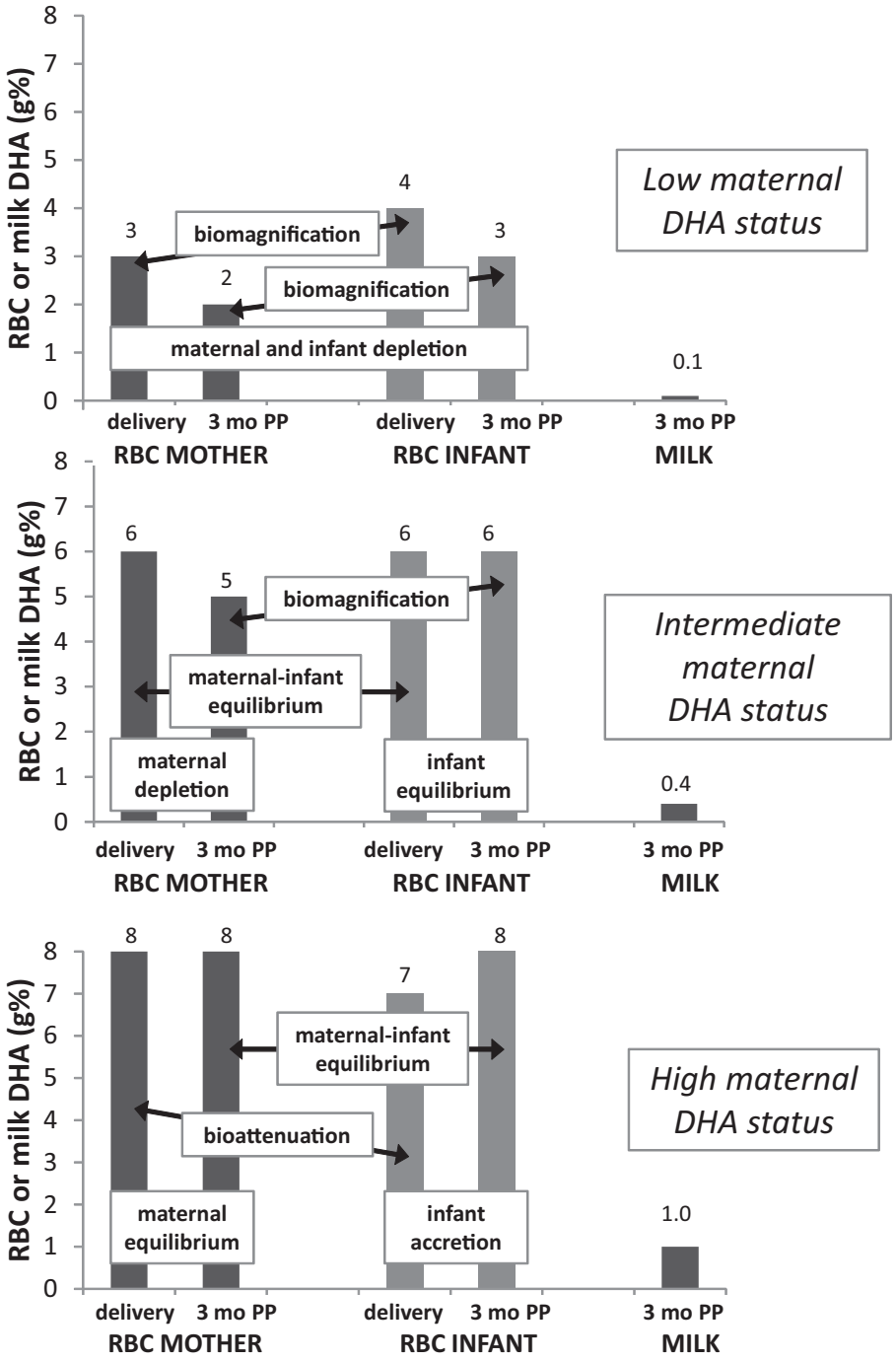

Figure 1. Relations between the maternal and infant erythrocyte (RBC) docosahexaenoic acid (DHA) content at delivery and 3 months postpartum at low, intermediate and high maternal DHA status and the concomittant milk DHA status. From biomagnification to bioattenuation.

this infant equilibrium was clearly associated with a decrease of the maternal DHA status during lactation.

4.2 Postpartum biomagnification and maternal DHA equilibrium: 8 g% RBC-DHA

Although the RBC-DHA content decreased consistently in all women during 3 months of lactation, the Maasai women showed the most, and the Sengerema women the least, pronounced decreases. Similarly, the absolute difference between the maternal and infant DHA status at delivery and at 3 months postpartum decreased in the order Maasai>Pare>Sengerema, with the noteworthy remark that Sengerema infants had a *lower* DHA status compared to their mothers at delivery (**Chapter 4.1**), but not at 3 months postpartum. In a subsequent analysis, **Chapter 4.2,** we used these observations, to calculate the *postpartum* turning-point from DHA biomagnification to bioattenuation. Secondly we estimated from which RBC-DHA content at delivery the lactating mother reaches a postpartum DHA-equilibrium, i.e. the steady state level from which the maternal RBC-DHA content discontinues to decline during lactation. We also investigated what would happen concomitantly to the infant RBC-DHA content. Finally, we investigated the relation between RBC-DHA and RBC-AA in these three populations with seemingly different intakes of both DHA and AA from local food sources.

We found that after 3 months lactation, the maternal RBC-DHA was lower than the corresponding infant RBC-DHA content up to a maternal RBC-DHA content of 8 g% at delivery. In other words, at the stable dietary habits observed in these populations, mothers with an RBC-DHA content of 8.0 g% at delivery had a similar RBC-DHA content at 3 months postpartum. However, this maternal RBC-DHA equilibrium corresponded with an infant RBC-DHA of 7 g% at delivery that increased to 8.0 g% at 3 months postpartum (Figure 1). The latter indicates that the initial bioattenuation of DHA during pregnancy in women with high fish intakes, is followed by a postpartum surge of DHA via the milk. The fact that biomagnification seems to occur up to 6 g% during pregnancy but up to 8 g% after delivery suggests that DHA becomes increasingly important for the infant directly after delivery. We also found between-group differences in maternal RBC-AA. Despite the observed differences in maternal RBC-DHA and RBC-AA, however, we found no differences in infant RBC-AA at delivery or 3 months postpartum, suggesting that unlike RBC-DHA, maternal RBC-AA and infant RBC-AA are independently regulated in these populations. We finally observed bell-shaped relations between RBC-DHA and RBC-AA, which might (in concert with the processes of biomagnification and bioattenuation) aim at uniform infant AA status. At low DHA status DHA biomagnification might synergistically increase AA status, while at high DHA status, DHA bioattenuation might antagonistically decrease AA status.

4.3 A bell-shaped relation between DHA and AA

To confirm the bell-shaped relation between DHA and AA that was found in **Chapter 4.2**, we investigated these suggested synergistic and antagonistic relationships in our FA databases composed of 1,979 RBC, 789 umbilical artery wall and 785 umbilical vein wall samples that had been

collected during 25 years of previous studies and which consisted of populations with a wide range of dietary AA and EPA+DHA intakes, **Chapter 4.3**. We found that in all compartments, but notably in RBC, the relation between EPA+DHA and AA appeared bell-shaped. Most evidently, populations with low RBC-(EPA+DHA) (i.e. <2 g%) exhibited positive relationships, while in populations with high RBC-(EPA+DHA) (i.e. >8 g%) we found negative relationships. Synergism between RBC-(EPA+DHA) and RBC-AA was also apparent in umbilical arteries and veins, but antagonism could not be demonstrated. The latter might be explained by our earlier observation that bioattenuation prevents by some mechanism the accumulation of DHA in the fetus at high maternal RBC-(EPA+DHA) content, so that an infant RBC-(EPA+DHA) content >6 g% at delivery is rarely observed. We speculated that synergism and antagonism aim at a certain LCPω3/LCPω6 balance that might be involved in optimal homeostasis and is favorable for 'good health'. We suggest that synergism might be a feature of the typically low LCPω3 status as observed in developing countries with very low LCPω3 intakes such as in the Maasai -where fish are considered as inedible- while the antagonistic suppression of AA at high RBC-(EPA+DHA) might be the physiological standard for human adults. This antagonistic relation is likely to have occurred in our ancient human ancestors who had both high intakes of AA and EPA+DHA from their combined terrestrial and aquatic food sources.

5 Brain, liver and adipose tissue FA compositions in an East African population with high fish intakes

In **Chapter 5** we present the brain, liver and adipose tissue FA compositions of samples collected from fetuses who were stillborn to women living on the Lake Victoria coastlines around Sengerema (see Scope, Figure 1) and were known to have high intakes of freshwater fish. We compared the outcomes with our findings for the adipose tissue FA compositions (**Chapter 6.1**) and the reconstructed FA compositions of liver and brain (**Chapter 6.2**) of fetuses born to women with typically Western diets. We analyzed the brain (n=18), liver (n=14) and adipose tissue (n=11) FA compositions deriving from 20 stillborn infants of different gestational ages (range 8-38 weeks) born to Tanzanian women with low LA intakes and high intakes of DHA and AA from local fish.

We found that with advancing gestation, the relative amounts of brain SAFA, PUFA, DHA and 20:3ω6, 22:4ω6 and 22:5ω6 increased, while MUFA, 20:3ω9, 22:3ω9 and AA decreased. The decrease in brain AA might be secondary to an augmented AA-metabolism that results in increases of 20:3ω6 (the precursor of the series-1 prostaglandins), 22:4ω6 (the main storage form of AA in the brain) and 22:5ω6. In the liver, SAFA, PUFA and LA increased, while MUFA decreased with gestation. In agreement with our earlier findings, the steep increase of (mostly *de novo* synthesized) SAFA in adipose tissue caused relative decreases of MUFA, PUFA, DHA, LA and AA with advancing gestation. Compared to Western infants, the currently studied African infants had higher DHA, lower AA, and a higher DHA/AA-ratio in brain and adipose tissue, while their LA content in adipose tissue was lower. We concluded that the low LA and high DHA and AA intakes of these African women might support optimal ALA vs. LA competition for fatty acid desaturase enzymes 1 and 2 (FADS1 and FADS2) and

DHA vs. AA antagonism. Conversely, the Western diet, characterized by high LA and lower DHA and AA intakes, might disturb these evolutionary conserved mechanisms aiming at an optimal ω3/ω6-balance.

Fetal body fatty acid composition in Western populations

6.1 Fetal adipose tissue fatty acid composition

Chapter 6 describes the FA composition of the fetal body. There is a scarcity of data that allow calculation of the fetal whole body biochemical composition during gestation and at delivery. Some investigators have examined the different components in detail but an accurate estimate of the total amounts of FA in the fetal body at the various times during gestation is missing. These data might be important, since FA and especially LCP have been implicated in fetal health and neurodevelopment. Some data are available for the FA compositions of the fetal liver and brain. It has been suggested that the majority of fetal LCP are not located within these organs but rather within the fetal adipose tissue compartment. There are very few investigators who reported on the FA composition of fetal adipose tissue during intrauterine development. We aimed at the elucidation of the remarkable discrepancy between the outcomes of the only two studies on the fetal adipose tissue FA composition that to our knowledge have been reported so far.

To gain insight into the intrauterine courses of FA accretion in fetal white adipose tissue (WAT) in Western infants we measured the WAT-FA compositions of 40 fetuses and newborns with gestational ages between 22-43 weeks **(Chapter 6.1)**. The samples were collected as part of a perinatal mortality study in Curaçao (the former Netherlands Antilles). We found that the fetal adipose tissue SAFA and MUFA contents increased from 83 to 298 mg/g WAT and 83 to 226 mg/g WAT, respectively, while PUFA increased insignificantly from 18.0 to 23.2 mg/g WAT. Consequently, SAFA increased from 46 to 55 g% of total FA, while MUFA and PUFA decreased from 44 to 41 g% and 10.3 to 4.26 g%, respectively. The infant adipose tissue DHA content decreased from 1.18 g% at 25 weeks to 0.30 g% at delivery, while AA decreased from 2.06 g% at 25 weeks to 0.66 g% at delivery. These data clearly demonstrated decreasing relative adipose tissue LCP contents, as a result of the increasing amounts of SAFA and MUFA that become stored. These declines had not been noted in a previous study[7] in which fetal FA accretion rates during gestation were estimated. The reported LCP accretion rates in that study and subsequent reviews[8-11] are much higher than presently calculated. To arrive at more realistic accretion rates, we calculated that at term, an appropriate for gestational age (AGA) infant harbors about 560 g lipid. A large for gestational age (LGA) infant of 4,000 g contains about 810 g lipid, while a 4,500 g LGA has about 1,060 g lipid. Analogously, an AGA infant contains about 1,460 mg DHA and 3,150 mg AA in its adipose tissue, while a 4,500 g LGA infants may have deposited in its adipose tissue up to 2,970 mg DHA and 6,420 mg AA. As a result, adipose tissue accretion rates during the third trimester for DHA increase from 88 mg DHA/week for a 3,500 g AGA infant to 184 mg DHA/week for a 4,500 LGA infant. Accretion rates for AA during the 3rd trimester increase from 193 mg AA/week for a 3,500 g AGA infant to 402 mg AA/week for a 4,500 LGA infant. Finally,

increment rates during the last 5 weeks of gestation were about 2-fold higher compared to the rates during the 3rd trimester. The current WAT-DHA and WAT-AA accretion rates are considerably lower than previously reported in the literature.

6.2 Fetal whole body LA, AA and DHA contents and accretions in pregnancy

There is no information on the whole body FA contents of preterm infants, although scattered information on the FA-composition of many organs is available. We therefore performed a second study, described in **Chapter 6.2**, to estimate whole body amounts of LA, AA and DHA and whole body accretion rates during intrauterine fetal development. We collected data on the weights, lipid contents and FA-compositions of the quantitatively most important fetal organs (i.e. skeletal muscle, skeleton, skin, liver, brain and adipose tissue) of appropriate for gestational age (AGA) Western fetuses. Subsequently, we estimated the total body contents of LA, AA and DHA at 25, 35 and 40 weeks of gestation.

We found that Western infants accrete FA in the order of LA>AA>DHA at all stages during pregnancy and that the highest accretion rates are reached in the last 5 weeks of gestation, i.e. 342 mg LA, 95 mg AA and 42 mg DHA/day. At term, most of the infant's LA, AA and DHA is located in adipose tissue (68, 44 and 50%, respectively). Substantial amounts of LA are also located in skeletal muscle (17%) and skin (13%); while important amounts of AA are located in skeletal muscle (40%) and brain (11%); and of DHA in brain (23%) and skeletal muscle (21%). At term age, an AGA infant has accreted about 21 g LA, 7.5 g AA and 3 g DHA, which constitutes a gap of 12 g LA, 3.3 g AA and 1.5 g DHA compared to a 35 weeks old AGA premature infant. We concluded that the presently estimated fetal LA, AA and DHA pool sizes and accretion rates may be useful to estimate the preterm infant's minimal requirements and the maternal LCP needs during pregnancy. However, since these data derive from populations with typically Western diets they do not necessarily reflect 'optima' for 'health'.

INSULIN SENSITIVITY AND FATTY ACID STATUS INTERRELATED

In **Chapter 7** we related insulin sensitivity to FA metabolism. The relation between insulin sensitivity and hepatic FA synthesis, i.e. *de novo* lipogenesis (DNL), has been studied intensively. High insulin levels are known to increase hepatic DNL. More recent studies showed that certain LCP, notably DHA, are strong suppressors of hepatic DNL. Lipogenesis might be important in some instances, i.e. secondary to the decreased insulin sensitivity in late pregnancy, but might be detrimental in diseased conditions, such as in diabetes and the metabolic syndrome, leading to non alcoholic fatty liver disease.

7.1 Higher DNL fatty acids in umbilical vessels during preeclamptic pregnancy

Preeclampsia is characterized by a combination of hypertension and proteinuria. The poorly understood etiology involves superficial placentation, maternal insulin resistance, dyslipidemia,

endothelial dysfunction, augmented systemic vascular resistance, platelet aggregation and coagulation. In a previous study, we found lower contents of both LCPω3 and LCPω6 in umbilical vessel walls of preeclamptic women living in Curaçao. In **Chapter 7.1** we investigated whether a similar picture would emerge in Tanzanian women living in Mwanza (see Scope, Figure 1) with high LCPω3 and LCPω6 intakes from Lake Victoria fish. We compared FA compositions of umbilical veins and arteries from 28 preeclamptic women with those of 31 normotensive non-proteinuric controls. In our preeclampsia cases we found lower DHA, LCPω6 and LCPω3+ω6 and higher SAFA and potentially *de novo* synthesized FA in umbilical veins in conjunction with higher 16:1ω7, ω7 and 18:0 in umbilical arteries. Interestingly, umbilical vessels in Mwanza preeclampsia cases showed higher DHA, LCPω3 and ω3 compared with the controls in Curaçao. We conclude that, both in Tanzania and Curaçao, preeclampsia is characterized by lower LCP and higher potentially *de novo* synthesized FA. We suggest that the similarity of the umbilical vessel FA abnormalities compared to controls in preeclamptic and diabetic pregnancies points at insulin resistance as a common denominator.

7.2 High DNL fatty acids in fetal and maternal RBC in late pregnancy

While investigating the milk- and RBC-LCP contents of the three Tanzanian populations (**Chapters 3 and 4**) with different LCP intakes, we did not pay much attention to the changes observed in those FA that potentially derive from hepatic DNL. In **Chapter 7.2**, we investigated the postpartum changes in DNL derived FA in maternal RBC. We focused on their relation with the well-known lower maternal insulin sensitivity at delivery as compared to the restored maternal insulin sensitivity at 3 months postpartum. Simultaneously, we investigated whether the observed FA changes between the three study populations from delivery to 3 months postpartum might relate to their differences in LCP, and notably DHA, intakes.

We found that the proxies for DNL activity decreased after delivery. At the same time, the proxy for the enzymatic activities of stearoyl-CoA desaturase (SCD, involved in desaturating SAFA to MUFA) decreased, while the proxy for elongation of very long-chain FA (Elovl) seemed to increase. The most important postpartum increases in maternal RBC-FA were on the account of 18:0, LA and AA, while the most important decreases were found for 16:0, 18:1ω9 and DHA. For infants the most important increases were for 18:1ω9, 22:5ω3 and LA, while decreases were noted for 16:0 and AA. We suggested that the increases in 18:0, and decreases in 16:0 and 18:1ω9, might derive from the postpartum reducing insulin-promoted DNL-activity, causing a more reduced SCD- than Elovl-activity. As a consequence of the latter, 16:0 increases, but also leaves more 16:0 for conversion to 18:0. A second finding was that the postpartum changes in the sum of potentially DNL-derived FA, as well as the sums of SAFA and MUFA, related negatively to RBC-DHA. This is in line with the described suppression of both SCD- and Elovl-activities by DHA. This influence is most likely conferred through sterol regulatory element-binding proteins (SREBP). We concluded that perinatal changes in maternal and infant FA status may be strongly driven by changing insulin sensitivity and also by DHA status, which concertedly may aim at the transport of certain nutrients across the

placenta or into the human milk at the various periods of infant development.

7.3 Higher DNL fatty acids in term compared to preterm colostrum milk

In a simultaneous study, **Chapter 7.3**, we related insulin sensitivity to changes observed in milk FA from colostrum to mature milk and to FA differences between preterm and term colostrum milk. We compared our data from **Chapter 2** with those in the literature and confirmed that preterm colostrum generally contains higher MCSAFA and LCP, and lower MUFA. With advancing lactation, MCSAFA generally increase, while both MUFA and LCP decrease. We investigated whether these changes, again, might be related to restoring maternal insulin sensitivity after delivery and to different magnitudes of maternal insulin sensitivity at the time of preterm and term deliveries.

We found a higher index for *de novo* synthesized FA in the breast, a lower index for *de novo* synthesized FA from the liver and lower SCD-activity in preterm compared to term colostrum. In both preterm and term milks, FA derived from mammary DNL increased with advancing lactation, while FA from liver DNL, SCD and FADS1 and FADS2 activities decreased. From these data we concluded that DNL in the breast is likely to be secondary to its lipoprotein lipase (LPL)-mediated uptake of FA from the circulation, which consists mainly of FA from hepatic DNL and adipose tissue lipolysis. This might explain lower MCSAFA (from mammary gland DNL) and higher MUFA (from insulin-induced hepatic-DNL) in term colostrum compared to premature colostrum, and also the decreasing MUFA, but increasing MCSAFA, with advancing lactation. We also suggested that MUFA and PUFA might compete for incorporation into very low density liprotein (VLDL). This might cause the high DHA content of preterm colostrum, secondary to a lower VLDL-MUFA content derived from hepatic DNL The decrease of milk LCP with advancing lactation seems to be explained by decreasing hyperlipidemia, LPL- and FADS-activities and an increasing MCSAFA milk content. We suggested that preterm delivery might thus deprive the infant from increasing transplacental MUFA and LCP transfer and cause premature exposure to the high PUFA and MCSAFA contents of the milk. In conclusion, the milk FA composition might be strongly influenced by the maternal magnitude of insulin sensitivity at the time of delivery and the subsequent restoration of insulin sensitivity after delivery.

8.1 Saturated fat, carbohydrates and cardiovascular disease

Chapter 8, finally, deals with the reigning paradigm that fat, especially SAFA, is unhealthy since its consumption is positively associated with cardiovascular disease (CVD) risk. This paradigm derives from the positive relation between the intake of dietary SAFA and serum cholesterol and the association between serum cholesterol levels and CVD[12-15]. In a recent meta-analysis[16] of 21 previously conducted prospective cohort studies, however, it was concluded that SAFA are not associated with CVD. It was shown that replacing dietary SAFA with CHO, notably those with high glycemic indices, was associated with an increase in CVD risk in observational cohorts, while replacing SAFA with PUFA was associated with reduced CVD risk[17,18]. Interestingly, replacing a

combination of SAFA and *trans*-FA with LA in controlled trials showed no indication of benefit but rather a signal towards increased CVD risk[19], suggesting that ω3-PUFA might have been responsible for the protective association between total PUFA and CVD.

We evaluated the roles of both CHO and SAFA in the etiology of CVD. High CHO intakes stimulate hepatic SAFA synthesis and also conservation of dietary SAFA since fat is spared during glucose excess. Hepatic DNL from CHO is also stimulated during *eucaloric* dietary substitution of SAFA by CHO with high glycemic index (GI) in normo-insulinemic subjects and during *hypocaloric* high-CHO/low-fat diets in subjects with the metabolic syndrome. The accumulation of SAFA stimulates chronic systemic low-grade inflammation through its mimicking of bacterial lipopolysaccharide and/or the induction of other pro-inflammatory stimuli. The resulting systemic low-grade inflammation promotes insulin resistance, reallocation of energy-rich substrates and atherogenic dyslipidemia that concertedly give rise to increased CVD risk. We conclude that avoidance of SAFA accumulation by reducing the intake of CHO with high GI is more effective in the prevention of CVD, than reducing SAFA intake *per se*. The combined results of this review support the contention that dietary CHO with high GI, notably in combination with dietary SAFA, give rise to an increased CVD risk, while LCPω3 reduce CVD risk.

MAIN CONCLUSIONS

9.1 Evolutionary niche, fatty acid intakes and ensuing fatty acid status

This thesis supports the contention that our Paleolithic ancestors evolved in a land-water ecosystem (**Chapter 1**). This ecological niche provided a diet rich in LCPω3 and LCPω6 (**Chapter 2**), which is reflected in the high milk (**Chapter 3**) and RBC (**Chapter 4**) DHA and AA contents of East-African women living in a land-water ecosystem. At the same time, the reconstructed Paleolithic diet was low in LA, which also became reflected in the low milk LA contents of women belonging to certain tribes with low intakes of refined vegetable oils (**Chapter 3**). In addition, African infants have higher whole body DHA (**Chapter 5**), but lower whole body LA and AA compared to Western infants (**Chapter 6**). Lower LA and AA together with higher DHA might support optimal ALA vs. LA competition for FADS1 and FADS2 enzymes and DHA vs. AA antagonism, while the Western diet, characterized by high LA and low DHA and AA intakes, might disturb these evolutionary conserved mechanisms aiming at an optimal ω3/ω6-balance.

9.2 Recommendations for 8 g% DHA in RBC and 1 g% DHA in milk

Taken together, the data of **Chapter 4** indicate that DHA biomagnification and bioattenuation aim at a certain fetal and infant DHA and AA status, but most importantly, that a maternal RBC-DHA above 6 g% supports a postpartum infant DHA equilibrium, while 8 g% seems sufficient to sustain both maternal and infant DHA status (Figure 1). This latter condition coincides with a mature milk DHA content of 1 g%, which may therefore serve as a target for infant formula. The relation between RBC-DHA and RBC-AA proved bell-shaped, suggesting that at low DHA levels, AA status

becomes adjusted by suppression, while at high DHA status both AA incorporation and synthesis become inhibited, leading to reduced AA status. Both these processes might be important in the regulation of the tight balance between the ω6 and ω3 series of LCP, which have both antagonistic and synergistic effects in e.g. metabolism, inflammation, blood pressure regulation and coagulation (**Chapter 4**).

9.3 Fetal DHA accretion rates in late gestation: 42 mg DHA/day

We confirmed that adipose tissue is the quantitatively most important location for LCP (**Chapter 6**). In large for gestational age infants and infants born to women with disturbed glucose tolerance, the expanding fetal adipose tissue compartment might compete with brain for incorporation of LCP. This Chapter also indicates that the previously reported infant LCP accretion rates were too high, because of the overestimation of the adipose tissue LCP content in a previous analysis with packed GC-columns. The estimated whole body increment rates of 95 mg AA/day and 42 mg DHA/day between 35 and 40 weeks gestation might provide information for the recommended maternal LCP intakes during pregnancy and the composition of infant formulas.

9.4 Compromised insulin sensitivity causes lower LCP status by dilution

The lower insulin sensitivity in late pregnancy becomes reflected by the FA composition of the umbilical cord vessels, RBC and breast milk (**Chapter 7**). The reduced insulin sensitivity in late gestation stimulates *de novo* lipogenesis (DNL), which is reflected by increasing amounts of DNL-derived FA in maternal and infant RBC, and in the milk around term birth. These DNL-derived FA subsequently decrease during lactation, concomitant with the restoration of insulin sensitivity. Comparably, the higher insulin sensitivity after a preterm compared to a term delivery is reflected by a lower content of DNL-derived FA in preterm milk. Similarly, umbilical vessels from preeclamptic pregnancies, in which reduced insulin sensitivity is a common denominator, contained higher DNL-FA compared to uncomplicated pregnancies (**Chapter 7).** These observations indicate that impaired insulin sensitivity becomes reflected by the FA compositions of most, if not all, body lipids. Thus, increased DNL might compromise a subject's LCP status by dilution, which might especially be noticeable in e.g. diabetic pregnancies, but might likewise has its influence in many other diseases characterized by impaired insulin sensitivity.

9.5 Not fat or SAFA, but intake of CHO with high GI relates to CVD

The current evidence suggests that SAFA and MUFA intakes are unrelated to CVD risk. Conversely, CHO, notably those with a high GI, and probably LA, increase cardiovascular disease risk, while ω3-PUFA are protective (**Chapter 8**). The positive relation between CHO and CVD is likely to relate to *de novo* lipogenesis and of sparing of SAFA in circulating lipids and tissues. It is thereby among the many environmental factors involved in chronic activation of the immune system by false triggers, causing chronic systemic low-grade inflammation, which in turn, results in insulin resistance,

altered glucose homeostasis and changes in the lipoprotein profile; all of which are hallmarks of the metabolic syndrome, which in turn is associated with increased risk of diabetes type 2, CVD and the many other degenerative diseases typical for Western societies (**Chapter 7**).

EPILOGUE

10.1 Evidence based medicine and evidence based nutrition

There is no doubt that prevention and treatment in the medical sciences should be supported by solid evidence. The strength or solidness of evidence is nowadays evaluated by the creation of a hierarchy in the outcomes of the various study designs (ranging from expert opinion to meta-analyses of randomized controlled trials) that has become part of what is nowadays commonly known as 'evidence based medicine' (EBM). EBM was first defined as 'a systemic approach to analyze published research as the basis of clinical decision making'. In 1996, Sackett et al.[20] more clearly defined EBM as 'the conscientious and judicious use of current best evidence from clinical care research in the management of individual patients'[21]. In practice, randomized placebo-controlled trials (RCTs) and notably meta-analyses thereof are considered as the highest level of evidence. In nutrition, a similar approach has recently been introduced and is known as evidence based nutrition (EBN).

10.2 This thesis in the light of evidence based nutrition

EBN is much more difficult to apply in decision making than EBM with e.g. drugs[22], although the latter is also blessed with difficulties in their own right[23-26]. Above all, there is no evidence for EBM, nor for EBN, as giving rise to better or more reliable results than other approaches, which actually labels the concept with the status of a 'paradigm**'. While people eat whole food or food products, EBN has to a large extent become the performance of RCTs with single nutrients. RCTs with single nutrients are, however, hampered by: poorly known dose-response relationships; multiple mechanism of action of a single nutrient; small dietary changes and influences that may induce pathology in the very long term; the many nutrient interactions; the ethical limitations that limit differences between intervention and control groups; the difficulty of patenting outcome results; and the inevitable problem that at e.g. isocaloric intakes any macronutrient always has to be replaced by another, which makes it precarious to attribute observed effects either to introduction of one macronutrient or the omission of the other[22,27].

RCTs with single nutrients and hard endpoints have nevertheless become the sole basis of many recommendations and guidelines by health authorities[28], while there is more concern for toxicity than for adequacy by the employment of the precautionary*** principle[29-31]. Moreover, in practice

** A set of assumptions, concepts, values, and practices that constitutes a way of viewing reality for the community that shares them, especially in an intellectual discipline.

*** The precautionary principle or precautionary approach states that if an action or policy has a suspected risk of causing harm to the public or to the environment, in the absence of scientific consensus that the action or policy is harmful, the burden of proof that it is *not* harmful falls on those taking the action.

the criterion of RCTs with hard endpoints for the preparation of nutritional recommendations is inconsistently applied. For example, there are no such RCTs supporting either the adverse effects of dietary cholesterol, SAFA, *trans*-FA or the beneficial effects of the consumption of 5-10 en% linoleic acid[19], while within the wealth of non-RCT information on vitamin D, only solid RCTs are eligible for the establishment of vitamin D adequate intakes[25,32]. In addition, all meta analyses of RCTs studying the role of LCP in neurodevelopment are negative[33-35], but there are nevertheless recommendations for their addition to infant formulas[36-38].

Most importantly, the currently reigning paradigm of EBN poorly appreciates the many interactions between dietary nutrients, ranging from synergistic to antagonistic health effects. The wide range of dietary ingredients creates a seemingly endless range of nutrients that need (expensive) testing before any recommendation can be made that derives from the highest level of evidence in EBN. It is high time for reconsidering what should be considered as 'evidence' in the nutritional research[39,40]. For this it has e.g. been suggested that nutrient policy decisions will have to be made using the totality of the available evidence[22], to make use of the Hill criteria[41,42] and to employ risk-benefit analyses[43].

The current thesis provides some evidence from the study of our ancient diet and the dietary patterns of traditional hunter-gatherers. It is conceivable that clues to narrow dietary nutrient ranges that need testing may derive from Evolutionary Medicine, since this discipline teaches us that our genome has been shaped during millions of years of evolution, during which time it has become slowly adapted to the contemporaneous conditions of existence, including our diet[44,45]. In the current thesis we focused mainly on FA, with some attention for the macronutrient composition of the ancestral diet. There are, however, at least 7 discrepancies between our current and Paleolithic diets, as depicted in **Chapter 1**, Figure 9. These include notably: a different FA composition (including a grossly decreased ω3/ω6 FA ratio, the combining of high intakes of SAFA with CHO, introduction of industrially produced *trans*-FA, reduced intakes of LCPω3 and LCPω6); a different macronutrient composition; a lower fiber content; a disbalanced Na-K ratio, a disbalanced acid-base balance (low vegetables/fruits and high CHO); a low micronutrient density (e.g. by reduced exposure to sunlight, low vegetables/fruits and high CHO); and a high glycemic load (CHO with high glycemic indices)[45,46]. Together with other changes in our lifestyle, these dietary changes may well lie at the basis of many, if not all, diseases that are typical for affluent countries. Further studies on the reconstruction of our ancestral diet will shed more light on the existing discrepancies with our current diet and thereby provide further clues for the testing of different diets, not single nutrients, by RCTs.

10.3 Evidence based medicine, eminence based medicine and biased evidence

There is good evidence that the diet of our earliest ancestors was rich in nutrients from the land-water ecosystem, whereas fish consumption is generally limited and unpopular in Western countries, especially among younger generations. This Paleolithic diet was high in protein and fat, including SAFA, and low in CHO, when compared to the current Western diet and, more troublesome, also

when compared to the recommendations in many Western countries. Contrary to various health campaigns (e.g. in The Netherlands: Let op Vet; Verzadigd = Verkeerd) that have been promoted by health authorities[47,48]. and the ensuing public opinion, it now seems that dietary fat and SAFA intake are unrelated to cardiovascular disease (Chapter 8). On the other hand, CHO, and notably those with high glycemic indices were shown to increase cardiovascular disease risk.

Thus, in retrospect, the previous 30 years of public campaigns to reduce fat, and notably SAFA intakes, have been successful in reducing these nutrients; but the net effect of the simultaneously increasing intakes of CHO, notably those with high glycemic indices, might actually have been an increased incidence of cardiovascular disease. The reconstructed Paleolithic intakes of SAFA contrast with the current recommendations to keep SAFA intakes below 10 en% or 'as low as possible'. The positive relation between CHO and cardiovascular disease may in part be explained by the observation that not SAFA intake *per se*, but rather carbohydrate-induced hepatic *de novo* lipid synthesis result in high circulating SAFA, while under these conditions the dietary SAFA are spared from oxidation. The *de novo* synthesized and spared SAFA may jointly be among the mediators of low-grade inflammation, which has emerged as a major cardiovascular disease risk factor. This notion is in line with the beneficial effects observed in the few trials that have thus far investigated the health effects of a Paleolithic-like diet that was low in refined CHO, while it contained considerable amounts of both protein and fat, including SAFA.

The fat contained within the Paleolithic diets is high in PUFA, notably ALA and LCP from the ω3-series, but low in LA. The inverse relation between LCPω3 intakes and cardiovascular disease risk is nowadays widely acknowledged and has been translated into the recommendation to eat (fatty) fish at least 2 times/week. However, the concurrent recommendation to reduce the consumption of fat is likely to reduce the SAFA intake, but also that of LCP. The current evidence that supports the health effects of an increased PUFA intake,was recently translated into a recommendation to increase LA intakes by a committee of the American Heart Association[49]. Apart from its contrast with the low LA intakes by our ancestors, this recommendation is marginally supported by the current evidence from RCTs, since these clearly demonstrate favorable effects of PUFA of the ω3 series, while PUFA of the ω6 series, notably LA, show a borderline insignificant tendency towards an *increased* cardiovascular disease risk[19].

Another example derives from the LCP composition of human milk and infant formula in Western countries, which contrast with both the recommendations issued for (maternal) fish intake and the currently observed LCP levels in the milk of East-African tribes living in the land-water ecosystem. Similarly, the currently low LCP intakes and low LCP status observed in Western populations contrast with the acknowledged lowest incidence of cardiovascular and psychiatric disease in Western populations residing in the highest quadrants of LCP intakes and LCP status, and also with the high LCP status of our East-African ancestors who evolved in the land-water ecosystem. The mechanism underlying the lowest cardiovascular and psychiatric disease risk might relate to the disturbance of an evolutionary conserved equilibrium between LCPω3 and LCPω6 FA, resulting in a

decreased LCPω3/LCPω6 ratio as a consequence of the low LCPω3 intakes in combination with the very high intake of LA. Either directly or indirectly through their influence on e.g. insulin sensitivity; the currently observed low LCPω3 status, as well as the high carbohydrate and low protein intakes in Western countries might be at the basis of metabolic disturbances including hyperinsulinemia, hyperglycemia and hyperlipidemia that are symptoms of the metabolic syndrome and contributors to the current burden of cardiovascular diseases in 'civilized' countries.

Taken together we contend that the discrepancies between the current nutritional recommendations and the evidence from a variety of study designs and also the composition of the diet of our early ancestors deserve to be taken seriously. These discrepancies are to be considered in the preparation of guidelines, but more research should certainly be done. For this, research grants may have to become reallocated from nature (genetics) to nurture (environment) notably because more than 95% of our current diseases are acquired diseases with a primary environmental background[50]. We are, however, probably facing a long-lasting aftermath of the health campaigns that, based on little evidence, have been held during the past 50 years. It is also questionable whether nutritional boards are willing to reconsider their positions in what should be accepted as evidence and what should not. This is important since there might be a growing gap between official guidelines and the information distributed by both laymen and prominent investigators in the field who are not willing to comply with the outcomes of RCTs with hard endpoints as the only criteria for Evidence Based Medicine and who keep pointing at the inconsistency of reasoning. For example, the recent doubling of the recommended daily intakes for Vitamin D from 50 to 100 μg/day by the Institute of Medicine suggests that the previous recommendation had little scientific basis, since no additional reports have been published with regard to the toxicity of Vitamin D in the meantime. Moreover, there was a jump in vitamin D supplement sales after Oprah Winfrey suggested on her show that the actual adequate intake of vitamin D is five times the current recommended dietary intake (and the vitamin D supplement sales in de USA are still increasing).

10.4 Strengths, limitations and recommendations for future study designs

A strength of the current thesis is that we have attempted to reconstruct our Paleolithic diet using a holistic approach, involving several lines of evidence ranging from paleo-environmental science, comparative anatomy, biogeochemistry, archeology and anthropology to (patho)physiology and epidemiology. A second strength comes from the fact that the composition of our reconstructed Paleolithic diets have, to a great extent, been confirmed by the analysis of the milk and RBC-FA compositions of traditionally living East-African tribes. Moreover, the observed differences between these traditional hunter-gatherer diets and typically Western diets are in support of the health issues that are increasingly identified by achievements in the traditional field of the nutritional sciences.

Several limitations need to be addressed as well. First, the reconstruction of *the* Paleolithic diet is impossible, since there is no such thing as '*the*' Paleolithic diet, while solid evidence for the wide dietary ranges of our ancestors are likely to remain subject to dispute. Future studies will especially

need to address the boundaries of our dietary composition as a whole, since some of the current diseases of civilization might be secondary to dietary nutrient intakes that are either too high (e.g. LA) or too low (e.g. DHA), compared to the intakes during the major part of human (genome) evolution. A second limitation is the lack of accurate dietary assessments of the African study populations, which obscure dose-response relationships between the observed nutrient status and the dietary intake. Third, although studies in traditional African populations are notoriously difficult to perform and are hampered by logistical and ethical issues, confirmation of the present results in longitudinal studies and with e.g. stable isotopes would improve the validity of these data and the impact in nutritional science. Last but certainly not least, an important limitation is that diet is not the only environmental factor involved in the epidemiology of the current typically Western diseases of affluence. Other lifestyle factors, such as insufficient physical activity, lack of sleep, altered intestinal flora, environmental pollution and chronic stress are also involved and each of these have largely unappreciated interactions in their own right.

10.5 Applicability of an evolutionary approach

We are convinced that the currently applied multidisciplinary approach of Evolutionary Medicine contains the inherent capacity to provide important insight into the components of a healthy lifestyle, including the composition of a healthy diet. The application of this insight will not so much increase our life expectancy, but rather increase our number of years without chronic disease. We therefore strongly support 'Evolutionary Medicine' to gain a more prominent role in the Medical and Nutritional sciences by e.g. the design of nutritional intervention trials, as opposed to the mere study of drugs or single nutrients. Return to the environmental balance of the Paleolithic era as translated to the culture of the 21st century might be the only effective manner to arrive at 'healthy aging', but is likely to become strongly contested by the current believers in the paradigm of 'Evidence Based Medicine' and notably the outcomes of RCTs and their meta-analyses, which is, as explained, not the definition of 'Evidence Based Medicine', or at least not intended to be from its very start.

Similarly, the current gene-centric obsession to understand diseases and the ensuing hype for (expensive) genome wide association studies is largely misplaced and much more attention should be paid to the understanding of our 'conditions of existence' as the most powerful force in evolution and as already recognized by Darwin. We all harbor alleles that are involved in obesity and insulin resistance; but this does not mean that these are faulty genes that deserve to become cured or bombarded with drugs. Most, if not all, of these 'faulty alleles', often erroneously named 'disease susceptibility genes', were already present in the first *Homo sapiens*, some 200,000 years ago. We were all selected against a background where these alleles produced some evolutionary advantage, causing conservation of the corresponding traits, such as responses to scarcity, famine and infection. Rather than accusing these faulty genes, it would be more profitable to search for the precipitating faulty environment, which from a dietary perspective means more attention for the current dietary abundances and imbalances.

10.6 Conclusions

The present thesis supports the adherence to the diets that have been consumed by our ancestors during the greater part of our evolution, notably the Paleolithic era stretching from 2.5 million to 10,000 years ago. Such a diet is higher in protein, similar in fat, lower in CHO and therefore richer in micronutrients, i.e. vitamins and minerals, but also in other bioactive nutrients with functions that are largely unknown. In practice this diet is composed of abundant vegetables and fruits, as combined with animal material that notably derives from the land-water ecosystem. The latter is the ecological niche that our ancient ancestors have inhabited in the past and consequently has shaped our genome to what it currently still is. CHO with high glycemic indices, and high CHO intakes combined with SAFA are unfavorable, notably to those of us who have other lifestyle imperfections, such as insufficient physical activity, lack of sleep, altered bacterial flora and exposure to environmental pollution and chronic stress. All of these are among the many factors contributing to a state of chronic systemic low-grade inflammation that causes functional adaptations in our sensitivity to insulin, our serum lipoprotein profile and many others. Such adaptations are evolutionary conserved survival strategies that are intended for the short term but in a chronic state ultimately result in an increased risk of the degenerative diseases of affluence, including diabetes type 2, CVD, neuropsychiatric diseases and certain types of cancer. The quality of the ingested fats is to be shifted towards increased intakes of LCP, notably AA, eicosapentaenoic acid (EPA) and DHA. We criticize the currently recommended, high intakes of CHO and of refined vegetable oils containing ω6-PUFA, notably LA, which have not been part of our Paleolithic diet and which exhibit well-documented interference with our ω3-PUFA status[51]. Future trials with Paleolithic diets are needed. These should obviously comply with the culture of the 21st century. They should preferably also take into account the many other lifestyle factors that we have changed since the agricultural and notably the industrial revolutions. We hypothesize that the outcomes of these trials will reveal no more than that Darwin was right.

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CHAPTER 9

Samenvatting voor de geïnteresseerde en de meer dan geïnteresseerde leek

ABSTRACT

Introductie. Darwin beschreef twee krachten in de evolutie: "natuurlijke selectie" en de "leefomstandigheden"; waarbij zijns inziens de laatste het meest belangrijk is. Ons erfelijk materiaal (genoom) ondergaat een zeer langzame aanpassing aan de snel veranderende leefomstandigheden. Evolutionaire geneeskunde leert ons dat typische welvaartsziekten, zoals hart en vaatziekte, suikerziekte, bepaalde soorten kanker en degeneratieve hersensziektes, worden veroorzaakt door een "mismatch" tussen onze eigenhandig veranderende leefomstandigheden en ons oeroude genoom. Voor de preventie en behandeling is het nodig om de veranderingen van onze leefomstandigheden sinds de landbouw- en de industriële- revolutie te erkennen en om gedetailleerd inzicht in te krijgen in deze veranderingen.

Materialen en Methoden. We voerden een multi-disciplinaire literatuurstudie uit naar de leefomstandigheden van onze verre voorouders die leefden in Oost-Afrika. Met deze gegevens reconstrueerden wij de samenstelling van verschillende Oost-Afrikaanse voedingen in de Paleolithische tijd. Tevens verzamelden we melk, rode bloedcellen, navelstrengvaten en vetweefsel van traditioneel levende Oost-Afrikaanse volkeren en bepaalden hiervan de vetzuursamenstelling.

Resultaten. Onze literatuurstudie toonde aan dat onze jagende en verzamelende voorouders leefden in het land-water ecosysteem en dus tenminste een deel van hun voedsel uit deze niche betrokken. Ten opzichte van onze huidige Westerse voeding hadden ze o.a. een lagere inname van koolhydraten en linolzuur, maar hogere innames van eiwit en de typische visolievetzuren EPA en DHA. Ons veldonderzoek in Oost-Afrika ondersteunde een hoge inname van visolievetzuren uit de lokale land-water ecosystemen.

Conclusies. Verschillen tussen onze huidige Westerse voeding en die van onze Paleolitische voorouders dragen sterk bij aan het grote aantal jaren dat de Westerse bevolking leeft met chronische ziektes. De speurtocht naar antwoorden op de vraag hoe we deze situatie kunnen verbeteren wordt echter gehinderd door het huidige paradigma dat ons voorschrijft hoe studies naar de gezondheidsaspecten van onze voeding en leefstijl moeten worden uitgevoerd. Het lijkt simpel en dat is precies wat het is: om gezond oud te worden moeten we teruggaan naar de leefomstandigheden van de Steentijd, zoals vertaald naar de cultuur van de 21ste eeuw.

Vetzuren en menselijke evolutie:
Een bijdrage aan de Evolutionaire Geneeskunde

De voeding en de klinisch chemische en metabole karakteristieken van traditionele bevolkingsgroepen uit de Oost Afrikaanse land-water ecosystemen

De achtergrond van dit proefschrift: Evolutionaire Geneeskunde

Via de nieuwsmedia krijgen we vrijwel dagelijks informatie over nieuw ontdekte erfelijke "afwijkingen" waarbij niet zelden wordt gesuggereerd dat deze "afwijkingen" ten grondslag liggen aan de belangrijkste chronische ziektes waarmee we in de Westerse maatschappij te maken hebben, zoals suikerziekte (diabetes mellitus type 2), hart en vaatziekte, bepaalde vormen van kanker en degeneratieve ziektes van de hersenen, bijvoorbeeld de ziekte van Alzheimer. Vaak eindigt zo'n bericht met de boodschap die in essentie neerkomt op: "Het is momenteel nog te vroeg, maar over een aantal jaren zullen nieuwe geneesmiddelen op de markt verschijnen die deze *foute* genen tot de orde zullen roepen". Het betreft één van de vele voorbeelden van hoe hoog gespannen onze verwachtingen zijn van het onderzoek van ons genoom*. Ook van hoezeer we gefocusseerd zijn op het *genezen* van welvaartsziektes en hoe weinig we aan de preventie hiervan doen. Merkwaardigerwijs is nagenoeg iedereen het er eveneens over eens dat "Preventieve Geneeskunde" de beste vorm is van Geneeskunde. Het verrichten van genetisch onderzoek is echter "sexy" en daar hebben we veel geld voor over in de vorm van subsidie. Onderzoek naar preventie is daarentegen moeizaam: het proces op weg naar de bovengenoemde welvaartsziektes strekt zich uit over tientallen jaren en zolang wachten om te zien of een leefstijlverandering zijn nut bewijst is nagenoeg onuitvoerbaar en onbetaalbaar. Bovendien zijn interventies in onze leefstijl doorgaans niet te patenteren, treden we bij voorkeur niet teveel in de privacy van een ander, en lijden we aan de mismoedige gedachte dat "mensen toch niet willen afvallen en ook al niet willen stoppen met roken als je ze op de gevaren wijst".

De berichtgevingen over de ontegenzeggelijk indrukwekkende resultaten van het genetisch onderzoek gaan er in hun enthousiasme aan voorbij dat de leefomstandigheden ("conditions of existence") reeds door Charles Darwin (1809-1882) als de verreweg meest belangrijke factoren werden beschouwd in de evolutie. Darwin heeft ons geleerd dat de evolutie gedreven wordt door de selectie ("survival") van dié individuen en soorten die het best zijn aangepast ("fittest"; dus nadrukkelijk niet een synoniem van het Nederlandse "fit") aan hun omgeving zodat zoveel mogelijk nageslacht kan worden voortgebracht. De primaire invloed van ons erfelijk materiaal in bovengenoemde typisch Westerse "welvaartziektes" is dan ook gering: de meeste, zo niet alle, met veel ophef aangekondigde, genetische "afwijkingen" in de mens waren reeds onder ons toen *homo sapiens* zo'n 160.000 jaar geleden ergens in Oost-Afrika ontstond en dus ook toen zo'n 100.000 jaar geleden de uittocht uit Afrika begon. Uitzondering vormen de zeldzame "aangeboren genetische

* Het genoom is ons totaal aan erfelijk materiaal (ons "DNA").

fouten", die minder dan 5% van alle ziektes veroorzaken. Daarentegen wordt geschat dat zo'n 90% van de patiënten met diabetes mellitus type 2, 80% van de patiënten met hart en vaatziekte, en 70% van de patiënten met een beroerte en met dikke darmkanker kunnen worden voorkómen als we meer aandacht besteden aan onze voeding, overgewicht, inactiviteit en roken. Het betreft de zogenaamde "verworven ziektes" waarbij de omgeving de primaire rol speelt. Uiteraard bestaan er tussen mensen genetische verschillen in de gevoeligheid voor de omgevingsfactoren die een rol spelen bij het ontstaan van welvaartsziektes. Het zijn deze *verschillen* in gevoeligheid die met veel ophef worden aangekondigd. Die ophef onttrekt onze aandacht van de primaire rol van de omgeving. Dat de oorzakelijke rol van 'foutieve' genen schromelijk wordt overdreven wordt pakkend weergegeven door de uitspraak dat "onze genetica het pistool laadt, maar dat het de omgeving is die de trekker overhaalt". Als we maar doorgaan met het veranderen van onze omgeving zal zelfs de meest zwaar afgestelde trekker uiteindelijk afgaan.

Algemeen wordt gesteld dat ons genoom sinds de Steentijd ("Paleolithische tijd": 2,5 miljoen tot 10.000 jaar geleden) nagenoeg niet is veranderd, hetgeen betekent dat we in essentie genetisch gelijk zijn aan onze voorouders die toen leefden. Gezien de snelheid waarmee de "typisch Westerse" ziektes zich hebben verspreid, en zich tegenwoordig verspreiden in de voormalige ontwikkelingslanden, zullen maar weinigen beweren dat in die korte tijd iets wezenlijks is veranderd in ons genoom. Het is dan ook op het conto van onze eigenhandige verandering van de omgeving dat de volstrekt normale genen die verantwoordelijk worden gehouden voor onze typisch Westerse ziektes momenteel niet meer goed functioneren. Dit wordt door wetenschappers die zich bezig houden met de "Evolutionaire Geneeskunde"** bedoeld als ze spreken over een slechte afstemming tussen de door onszelf veranderde omgeving (waaronder onze voeding) en ons oeroude genoom. Het hieruit voorkomende conflict begon met de landbouwrevolutie (ongeveer 10.000 jaar geleden) en is in een stroomversnelling geraakt sinds de industriële revolutie (zo'n 100-200 jaar geleden).

Wat weinig bekend is, is dat, afgelezen aan hun botten, de gezondheid van de mensen die de landbouwrevolutie begonnen aanvankelijk verslechterde. Dit verlies werd echter gecompenseerd door het feit dat deze landbouwers meer kinderen kregen dan de hen omringende jager-verzamelaars. Daarmee bood de omschakeling van jagen-verzamelen naar landbouw en veeteelt een netto evolutionair voordeel, ondanks de afgenomen gezondheid en levensverwachting. Een vergelijkbare paradox*** is ontstaan sinds de industriële revolutie. Het zijn vooral de hygiënische maatregelen, het terugdringen van infecties (o.a. kinderziekten), hongersnoden en oorlogen, een toegenomen welvaart en de behandeling van chronische degeneratieve aandoeningen ("ouderdomsziekten") die hebben geleid tot onze nog steeds toenemende levensverwachting. Wat hierbij echter zelden wordt belicht is dat er sinds de industriële revolutie een afname is van het aantal jaren dat we zonder chronische ziekte leven. De toegenomen levensverwachting sinds de industriële revolutie overschaduwt daarmee het toegenomen aantal jaren met chronische

** In de evolutionaire geneeskunde (in het Engels ook wel *Darwinian Medicine* genoemd) worden de ziekten van de mens verklaard vanuit de evolutietheorie.
*** Schijnbare tegenstelling

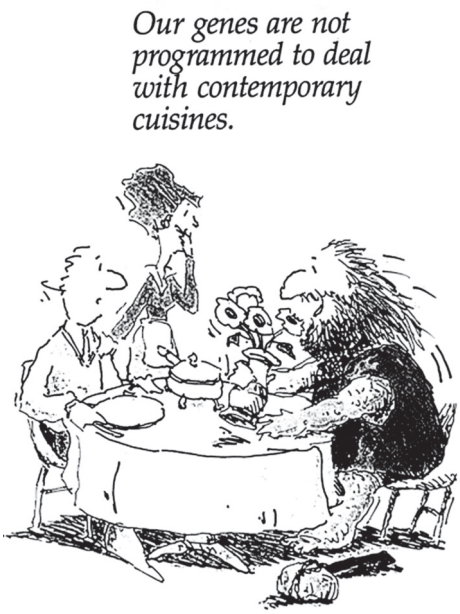
degeneratieve aandoeningen. Vanuit een evolutionair oogpunt zijn er nagenoeg geen nadelen aan deze ouderdomsziekten verbonden, omdat ze meestal optreden ver na het bereiken van de reproductieve leeftijd. Dit ondanks het feit dat de gemiddelde leeftijd voor het krijgen van kinderen in Westerse landen inmiddels is verschoven naar rond het dertigste levensjaar. Onderzoekers hebben berekend dat de eliminatie van alle risicofactoren voor de belangrijkste ouderdomsziekten de levensverwachting met slechts 4 jaar zal doen toenemen. Evolutionair geneeskundigen benadrukken dan ook dat de werkelijke winst is te behalen in een toegenomen aantal jaren in goede gezondheid zonder chronisch degeneratieve ouderdomsaandoeningen aan het eind van onze levenscyclus ("healthy aging"). Dit is niet alleen gunstig voor het betreffende individu, maar tevens de enige werkelijk effectieve manier waarop we de kosten in de Gezondheidszorg kunnen beheersen zonder de kwaliteit hiervan aan te tasten.

De consequenties van reductionistisch denken

Een tweede wijdverspreide misvatting is dat "de wetenschap het wel zal oplossen". Dat is, met alle bijbehorende tekortkomingen, ook inderdaad vaak het geval, tenminste als we het hebben over "behandeling". Maar in werkelijkheid weten we vanwege haar enorme complexiteit nog maar weinig van de ons omringende natuur en dat komt vooral treffend naar voren in het onderzoek van onze voeding. Dat is meer dan "jammer" omdat dit precies de discipline is die zich in hoge mate leent voor preventie. In de ons omringende natuur heeft alles met alles te maken, maar in ons onderzoek van deze natuur dienen we vanwege de beperkingen van de menselijke geest sterke vereenvoudigingen te hanteren, hetgeen ook wel met "reductionisme"**** wordt aangeduid. Om zeer goede redenen hebben we voor de interpretatie van de resultaten in Medisch onderzoek objectieve normen geformuleerd, hetgeen we "Evidence Based Medicine" noemen en in de Voedingswetenschap "Evidence Based Nutrition". Daarbij is het hoogste niveau van "bewijs" dat we kunnen bereiken het "gerandomiseerde onderzoek", waarbij de onderzochte personen in twee willekeurig gekozen subgroepen worden opgedeeld. De ene groep krijgt dan bijvoorbeeld een geneesmiddel en de andere een nepstof ("placebo"). Noch de proefpersonen, noch de onderzoekers, weten wie wat krijgt en na een afgesproken tijd kijken we welke groep nu het meeste baat had van de behandeling. In het gunstigste geval is de actieve stof beter dan het placebo. Deze benadering is uitermate geschikt voor het evalueren van een geneesmiddel, maar veel complexer ligt het als we te maken hebben met een stof die we uit de omgeving dienen te verkrijgen, zoals een voedingsstof. Want zo'n "nutriënt" staat immers nooit op zichzelf. Het is onderdeel van een systeem, want we eten niet die ene stof: we eten een stuk vlees, of een tomaat waarin deze stof een functie vervult. In die tomaat zitten miljoenen stoffen en die hebben allemaal met elkaar te maken. Ze maken onderdeel uit van een levend systeem, zoals wijzelf: onze voeding leeft! Momenteel zou je echter niet direct op die gedachte komen als je kijkt naar een witte boterham belegd met een plak vlees of besmeerd met

**** De tegenhanger van "reductionisme" heet in de biologie de "systeembiologie". Dit is de wetenschap die biologische systemen tracht te bestuderen in hun volledige complexiteit.

margarine en jam, want elk van deze samenstellende componenten staan, door o.a. het vetmesten van ons vee met granen in plaats van grassen en onze bewerkingen van de oorspronkelijke granen (malen, raffineren), olie bevattende zaden (persen, raffineren) en vruchten (bewerken, toevoegen suikers, conserveermiddelen), ver van de natuur. De Paleolithische mens zou het niet herkennen als iets eetbaars (**Figuur 1**).



Figuur 1. Dit Figuur sierde de kaft van het maandblad van de Medische Faculteit van de University of California San Francisco rondom mei 1986. Het verwees naar een lezing over onze oervoeding die in de UCSF was gehouden door Prof. Dr. S. Boyd Eaton.

Als je de effecten van een nutriënt in de mens wil bestuderen, dien je dat dus vanwege de door onszelf opgelegde normen***** in principe te doen volgens de regels van de "Evidence Based Medicine", dus via dat "gerandomiseerde onderzoek". Maar we kennen niet exact de dosering, laat staan dat we goed weten in welke verhoudingen de bestudeerde stof dient te staan ten opzichte van de miljoenen andere stoffen die deel uitmaken van dat "levende" systeem dat onze voeding is, of beter gezegd: onze voeding vroeger was. Ook vervult zo'n nutriënt in ons lichaam vele functies en dat is dus anders dan wat we willen met een geneesmiddel waarbij we meestal mikken op een enkele functie en niet de functies die je op de koop toe moet nemen (bijwerkingen). De vele hieruit voortkomende doseringen, combinaties van nutriënten en te onderzoeken functies zijn menselijkerwijs niet te bestuderen, nog afgezien van het feit dat dergelijk onderzoek niet is te betalen.

De oplossingen die de huidige Voedingswetenschap op basis van "gerandomiseerd onderzoek" met een enkel nutriënt levert draagt dan ook het gevaar in zich dat het vastgestelde gunstige effect van

***** Dit heet ook wel een "wetenschappelijk paradigma": een complex geheel van opvattingen, methoden en vraagstellingen, dat de wetenschappelijke gemeenschap van een bepaald tijdvak een idee geeft van wat de belangrijke vragen zijn, en hoe die opgelost moeten worden. Indien vanwege voortschrijdend onderzoek de wetenschappelijke inzichten plotseling veranderen spreekt men van een "paradigma shift".

die ene dosering van die ene stof (b.v. vitamine D) op de bestudeerde ziekte (b.v. osteoporose), ook gepaard gaat met bijwerkingen (b.v. meer nierstenen). Want als je hier drukt gaat ergens anders iets omhoog en wel op een plaats die je niet had verwacht. Misschien hadden we met de inname van meer vitamine D ook meer water moeten drinken, de inname van koolhydraten moeten verlagen en meer groente en fruit moeten eten, zodat er meer doorstroming was, meer vitamine K_2 werd gemaakt door de bacteriën in onze dikke darm, de urine minder zuur werd, we minder calcium gingen uitscheiden en uiteindelijk die nierstenen dus niet zo snel konden ontstaan. Dit voorbeeld is overigens verre van toevallig of irreëel, want de bovengenoemde combinatie van leefstijlfactoren waren inderdaad aanwezig in de vroegere tijd. We dienen dus een beetje een idee te hebben over wat de verhoudingen tussen onze voedingsstoffen dienen te zijn, hetgeen we ook wel aanduiden als "de balans". Het betreft de balans tussen onze voedingsstoffen, maar ook tussen de hoeveelheid voeding, fysieke activiteit, de mate van stress en het aantal uren dat we dienen te slapen. Al deze balansen liggen verankerd in onze evolutie zoals die plaats vond in een bepaalde omgeving en die ons gemaakt heeft tot wat we waren, maar nu niet meer zijn. Darwin leerde ons dat er eerst een omgeving was en dat daarop een aangepast organisme tot stand kwam, zoals bijvoorbeeld geïllustreerd door de verschillende snavels van zijn legendarische Galapagos vinken. Tijdens het allergrootste grootste deel van de evolutie op deze aarde was de omgeving er niet bij de gratie van de mens: *wij* zijn ontstaan bij de gratie van de omgeving, waarmee niet de huidige omgeving wordt bedoeld, maar de oeromgeving van onze verre voorouders.

Terug naar vroeger, zoals vertaald naar de normen van de 21ste eeuw

De snelle opkomst van Westerse ziektes leert ons dat we fouten hebben gemaakt in de bovengenoemde balans. Zoals gezegd, er zijn maar weinigen die zullen beweren dat in die korte tijd iets wezenlijks is veranderd in ons genoom. Vanwege de vele fouten die we hebben geïntroduceerd bestaat er dus ook niet één of andere "magic bullet", zoals "eet een beetje meer vis en dan komt alles wel goed". We zouden dus eigenlijk terug moeten naar de balans van het verleden, maar dan wel met het behoud van de cultuur van de 21ste eeuw. Daarbij dienen we de ongunstige leefomstandigheden van destijds te vermijden, zoals infectieziektes, honger en geweld. Vooral de uitbanning van deze ongunstige omstandigheden hebben, samen met de Gezondheidszorg, gemaakt dat we in de huidige Westerse maatschappij gemiddeld veel langer leven dan onze voorouders en de mensen in de huidige ontwikkelingslanden. We worden echter niet gezond oud, sterker nog, ons aantal jaren in gezondheid daalt en dat is nu precies waar het hier om gaat.

Het doel van dit proefschrift

Het doel van dit proefschrift is dan ook om een bijdrage te leveren aan het "gezond oud worden". Hierbij stellen we ons vooral de vraag hoe die balans in de leefstijl van onze voorouders nu eigenlijk was. In dit proefschrift hebben we getracht om bepaalde aspecten van de voedingsbalans te reconstrueren waarmee we in de evolutie zijn geworden wat we waren, maar wat we vanwege onze

eigenhandige veranderingen in de omgeving momenteel niet meer zijn. We focusseren vooral op vetzuren, hetgeen onderdelen zijn van het vet dat we eten, maar ook de vetten die we in ons lichaam zelf maken. Ons onderzoek volgde de gedachte van de Evolutionaire Geneeskunde, hetgeen een jonge discipline is die in zijn activiteiten gebruik maakt van de kennis van vele andere disciplines. Het betreft vooral enkele onderdelen van de klassieke Geneeskunde, zoals de Fysiologie (leer van de normale levensverrichtingen), Pathofysiologie (leer van de abnormale levensverrichtingen), Epidemiologie (leer van de ziekteverspreiding), Genetica (ons erfelijk materiaal) en de modernere Epigenetica (o.a. hoe onze genen worden aangedreven door de omgeving), maar ook disciplines die normaliter in de Geneeskunde minder intensief worden bestudeerd zoals de Evolutieleer, de vergelijkende Biologie, de Archeologie en de Antropologie.

Voor de meer dan geïnteresseerde leek: wat er in de diverse hoofdstukken staat

Concreet is in het eerste deel van dit proefschrift (**Hoofdstukken 1 en 2**) gewerkt aan de reconstructie van de voedingssamenstelling van onze Paleolithische voorouders. In het tweede deel (**Hoofdstukken 3-6**) doen we verslag van het onderzoek dat we deden aan mensen die er nog een (zo goed als) traditionele leefwijze op nahouden, zoals bijvoorbeeld de Hadzabe in Tanzania (Oost-Afrika), die behoren tot één van de laatste volkeren die nog jagen en verzamelen, en de Maasai, een tevens in Oost Afrika levend zeer traditioneel herdersvolk. In het laatste deel (**Hoofdstukken 7 en 8**) belichten we de mechanismen die ten grondslag zouden kunnen liggen aan de waargenomen verschillen.

De reconstructie van de voeding van onze voorouders uit de Steentijd is gebaseerd op berekeningen waarbij we zijn uitgegaan van een aantal jager-verzamelaar strategieën (b.v. het jagen op enkel landdieren of jagen-verzamelen in het land-water ecosysteem) van onze voorouders, een bepaalde fysieke activiteit en de samenstelling van de voedingsproducten zoals die deels in de literatuur beschreven staan, maar ook deels door onszelf zijn vastgesteld in het onderzoek in Oost-Afrika. Om een uitspraak te kunnen doen over de omgeving waarin onze voorouders opgroeiden voerden we eerst een uitgebreide literatuurstudie uit (**Hoofdstuk 1**). Vanuit verschillende disciplines, waaronder de wetenschappen van het Paleo-milieu, de vergelijkende Anatomie, de Archeologie, de Antropologie, de Pathofysiologie, de Epidemiologie en uit isotopen onderzoek, blijkt dat onze voorouders altijd dicht in de buurt van water hebben geleefd. Een opvallende conclusie van deze literatuurstudie is dan ook dat we momenteel weinig oog hebben voor de sterke aanwijzingen dat onze voorouders een deel van hun voedsel betrokken uit het water. Onze voorouders worden bij voorkeur voorgesteld als heldhaftige jagers, die met speren op grootwild jaagden en met vuistbijlen schedels en pijpbeenderen kraakten om aan hersenweefsel en beenmerg te komen. Dat er veel botten zijn gevonden met daarop de inkepingen en snijsporen van vuistbijlen betekent nog niet dat onze voorouders enkel op de grote graseters van de savanne jaagden. Daarom besluiten wij de literatuurstudie met de conclusie dat *tot het tegendeel bewezen is*, de voeding van onze voorouders, die in de nabijheid van het water leefden, ook bestond uit bijvoorbeeld vis en schelpdieren.

In het daaropvolgende hoofdstuk (**Hoofdstuk 2**) hebben we een reconstructie gemaakt van de Paleovoeding. Voor deze reconstructie hebben we gebruik gemaakt van de natuurlijke producten die, soms ook nu nog, in Oost-Afrika wijdverspreid verkrijgbaar waren voor consumptie. Het resultaat was een voeding met een hoger eiwit en vetgehalte, maar een lager koolhydraat gehalte in vergelijking met wat er tegenwoordig gegeten wordt door de "gemiddelde" Nederlander. De Paleovoeding lijkt voor wat betreft de hoge eiwit (gemiddeld 27 energie%, en%) en vet (34 en%) gehaltes en het lage koolhydraat gehalte (39 en%) nog het meest op het South-Beach dieet (eiwit 26 en%, vet 40 en% en koolhydraat 33 en%). Ook het Atkins dieet (eiwit 29 en%, vet 62 en% en koolhydraten 9 en%) is een hoog-eiwit voeding, maar in dit dieet gaat de hoge vetinname ten koste van de inname van groente en fruit, die o.a. rijk is aan koolhydraten, vitaminen, mineralen en vele andere bioactieve stoffen. Een duidelijk verschil met het Atkins dieet en bovendien met de huidige aanbevelingen van de American Heart Association (AHA) ligt in de kwaliteit van het gegeten vet. In het Atkins dieet ligt de nadruk op verzadigd en enkelvoudig onverzadigd vet, terwijl de AHA aanbeveelt om de inname van verzadigd vet onder de 7 en% te houden en 5-10 en% van het meervoudig onverzadigde vetzuur linolzuur (LA, o.a. aanwezig in zonnebloemolie) te consumeren. De gereconstrueerde Paleovoeding laat echter een relatief hoge inname van verzadigd vet (gemiddeld 12 en%) zien, met daarbij juist een lage inname (<5 en%) van LA, waarbij de lage linolzuur inname gecompenseerd wordt door een hoge inname van de "lange keten" meervoudig onverzadigde ω3-"vis"-vetzuren, zodat de inname van meervoudig onverzadigde vetzuren boven de 10 en% ligt. De berekende inname van deze "lange keten" meervoudig onverzadigde ω3-"vis"-vetzuren uit het Afrikaanse land-water ecosysteem is daarmee ongeveer gelijk aan wat de Eskimo's in Groenland hieraan eten en dat is ongeveer 100 maal meer dan wat de gemiddelde Nederlander hiervan at in 2003 (84 mg). Daarbij is het belangrijk om te weten dat een te lage inname van deze ω3-"vis"-vetzuren een intussen bewezen causaal verband heeft met hart en vaatziektes en met depressie, terwijl een te lage inname van de ω3-"vis"-vetzuren door pasgeborenen in verband wordt gebracht met een suboptimale ontwikkeling van de hersenen.

Om de biochemische effecten van de gereconstrueerde samenstelling van de Paleovoeding nader te evalueren, deden we onderzoek onder enkele traditioneel levende stammen die in Oost-Afrika nog aanwezig zijn. Die wonen in Tanzania en waren nog niet in aanraking gekomen met de Westerse voedselketens. Het overgrote deel van hun voedsel bestond uit lokale natuurlijke producten, waaronder met name veel groente, fruit en vis. In overeenstemming met de verwachting toonden wij in hun moedermelk (**Hoofdstuk 3**) hoge gehaltes meervoudig onverzadigde vetzuren aan. Terwijl vissen afkomstig uit de Noordzee voornamelijk meervoudig onverzadigde vetzuren uit de ω3-serie bevatten (met name eicosapentaeenzuur, EPA; en docosahexaeenzuur, DHA), bleken de Afrikaanse vissen uit zowel het zoet- als het zoutwater milieu lagere EPA gehaltes te bevatten. Naast hoge gehaltes DHA bevatten ze ook hoge gehaltes aan de ω6-vetzuren (met name arachidonzuur, AA).

In een daaropvolgende studie (**Hoofdstuk 4**) toonden we aan dat de hoge gehaltes van DHA en AA in de Afrikaanse vissen ook terug te vinden waren in de rode bloedcellen die we hadden geïsoleerd uit het bloed van gezonde proefpersonen. Het bleek dat de gemeten DHA waarden in de rode bloedcellen van sommige viseters vergelijkbaar waren met de door andere wetenschappers aangedragen optimale waarden voor de bescherming tegen psychiatrische aandoeningen en hart- en vaatziekten. De DHA inname van de moeders was in een bepaalde populatie zo hoog dat ze bij de geboorte van hun kinderen hogere DHA gehaltes in hun rode bloedcellen hadden dan hun pasgeboren kinderen. Deze opzienbarende nieuwe vondst staat daarmee in tegenstelling tot de situatie in Westerse vrouwen, die door hun lage DHA inname tijdens de zwangerschap interen op hun DHA voorraden. Bij de bevalling hebben de meeste Westerse moeders zelfs een lagere DHA status****** dan kun kinderen. Deze zogenaamde 'maternale DHA depletie' is in verband gebracht met het optreden van depressies tijdens en na de zwangerschap. Uiteindelijk bundelden we de uitslagen van alle door ons tot nog toe uitgevoerde onderzoeken onder vrouwen met verschillende innamen van DHA, om tot de conclusie te komen dat de relatie tussen sommige vetzuren niet zo eenduidig is als dat het aanvankelijk leek. Zo toonden we aan dat bij een lage DHA status, de AA status ook laag blijft. Als de DHA status stijgt, stijgt ook de AA status tot dat een constante AA status wordt bereikt. Echter als de DHA status nog verder stijgt wordt vanaf een bepaalde DHA status de AA status onderdrukt. Deze complexe relatie tussen DHA en AA speelt mogelijk een rol in de soms controversiële uitkomsten van de onderzoeken die tot nu toe met beide vetzuren zijn gedaan. De gevonden relatie is mogelijk belangrijk, omdat het onderdrukken van de AA status door het uit vis afkomstige EPA en DHA, ontstekingsreacties kan onderdrukken. Lang voortdurende lichte ontstekingsreacties worden in toenemende mate gezien als de belangrijkste *oorzaak* van het ontstaan van onze typisch Westerse ziektes, waaronder hart en vaatziekten, depressie en de ziekte van Alzheimer.

In **Hoofdstuk 5** hebben we in een studie van doodgeboren kinderen onderzocht of bij een hoogfrequente visinname door de moeder ook de hersenen, de lever en het vetweefsel een hoger gehalte van de eerder genoemde vetzuren AA en DHA bevatten. Zoals gezegd bevat de locale Afrikaanse vis hoge AA en DHA gehaltes. In vergelijking met studies die in het verleden onder Westerse kinderen waren uitgevoerd, vonden we in de hersenen en het vetweefsel van deze Afrikaanse kinderen hogere gehaltes DHA, maar juist iets lagere gehaltes aan AA. Dit komt dus overeen met de eerder in de rode bloedcellen en navelstrengen aangetoonde competitie tussen AA en DHA bij een hoge DHA inname. Bovendien vonden we in de Afrikaanse kinderen veel lagere gehaltes linolzuur (LA, de precursor van AA). LA zit voornamelijk in plantaardige oliën en het ziet er in toenemende mate naar uit dat de grote hoeveelheid LA in onze Westerse voeding mede verantwoordelijk is voor onze lage EPA+DHA status, omdat LA de synthese van EPA en DHA uit hun precursor (α-linoleenzuur; ALA) remt en ook omdat LA de inbouw van EPA en DHA in onze weefsels remt.

****** met status wordt bedoeld de totale hoeveelheid van een bepaalde nutrient die in het lichaam aanwezig is

In **Hoofdstuk 6** beschrijven we ons onderzoek naar de samenstelling van het onderhuidse vetweefsel van het kind tijdens de zwangerschap. Het onderhuidse vetweefsel dient als opslagplaats voor de eerder genoemde vetzuren zoals LA, AA en DHA. Vlak na de geboorte drinkt het kind nog maar weinig melk en sommige wetenschappers veronderstellen dat in deze belangrijke periode het vetweefsel van het kind niet alleen de belangrijkste bron is voor het opwekken van energie maar ook van de AA en DHA die nodig zijn voor de ontwikkeling van de hersenen. De hersenen ondergaan rondom de geboorte een enorme groei (de zogenaamde groeispurt) en dat vergt een forse aanvoer van AA en DHA. Ons onderzoek bevestigt dat het vetweefsel van het pasgeboren kind grote hoeveelheden AA en DHA bevat. Ook was al bekend dat gedurende de zwangerschap zowel de hoeveelheid vet in het kind alsook het vetgehalte van het vetweefsel (vetweefsel bestaat immers ook voor een groot gedeelte uit water) toeneemt. Wat wij vanwege ons onderzoek daarin kunnen nuanceren is dat er tijdens de aangroei van het vet in de baby eigenlijk geen verdere stijging van AA en DHA in het vetweefsel plaatsvindt, maar dat met name nog veel verzadigde vetzuren in het vetweefsel worden opgenomen. Eerdere onderzoekers hadden vastgesteld dat er weliswaar geleidelijk meer vet kwam, maar waren ervan uitgegaan dat het vet steeds dezelfde samenstelling bleef houden. Zowel de onverzadigde als de verzadigde vetzuren namen weliswaar in absolute hoeveelheden toe, maar doordat de verzadigde vetzuren wel 100 keer meer toenamen, werden de meervoudig onverzadigde vet zuren verdund. Door dit onderzoek beschikken we nu over betere schattingen van de totale hoeveelheden van de belangrijke vetzuren AA en DHA die een kind bij de geboorte in het vetweefsel heeft. Andere onderzoekers hadden al eens beschreven hoeveel AA en DHA zich bevinden in organen zoals de hersenen, de lever, de spieren, de huid en de botten van een pasgeboren kind. Door hun informatie met die van ons te combineren konden we uitrekenen hoeveel AA en DHA een pasgeboren kind in totaal bevat. Dit is bijvoorbeeld belangrijk bij het geven van flesvoeding aan prematuur (te vroeg) geboren kinderen, omdat nu kan worden ingeschat hoe groot hun tekort is aan AA en DHA in vergelijking met een a term (op tijd) geboren kind.

In **Hoofdstuk 7** gaan we verder in op de onderliggende mechanismen van onze bevindingen zoals beschreven in Hoofdstukken 2 en 3. Daar zagen we namelijk dat er vaak grote verschillen waren tussen de vetzuursamenstelling van de rode bloedcellen van zwangere vrouwen en niet-zwangere vrouwen, en ook tussen mensen die veel en weinig vis aten. En in het laatste geval waren de verschillen niet enkel terug te voeren op verschillen in de gehaltes van vetzuren die afkomstig zijn uit de vis. Om te onderzoeken hoe deze verschillen konden worden verklaard moesten we dieper ingaan in de mechanismen die we gebruiken voor het maken van vetzuren in ons lichaam. In dit hoofdstuk richtten we ons juist niet op AA en DHA, maar op de andere vetzuren, namelijk de al eerder genoemde verzadigde vetzuren (b.v. palmitinezuur, dat veel in dierlijk vet zit), maar ook op de enkelvoudig onverzadigde vetzuren (b.v. oliezuur, dat veel in olijfolie zit). De verzadigde en enkelvoudig onverzadigde vetzuren kunnen we, in tegenstelling tot de meervoudig onverzadigde vetzuren zoals LA, AA en DHA, zelf maken uit stoffen zoals glucose. We kunnen ze echter niet alleen uit suikers maken, maar bijvoorbeeld ook uit brokstukken van AA en DHA die ontstaan als de lever

deze afbreekt. Met andere woorden, de belangrijkste fabriek in ons lichaam: de lever, kan wel verzadigde en onverzadigde vetzuren maken uit suikers en andere stoffen, maar kan dat niet voor AA en DHA.

Wat we in Hoofdstuk 7 lieten zien is dat de zwangere vrouw meer verzadigde en enkelvoudig onverzadigde vetzuren leek te maken dan de niet-zwangere vrouw. Dit zou een logische aanpassing zijn om ervoor te zorgen dat haar groeiend kind tijdens de zwangerschap veel vet via de placenta krijgt aangevoerd. Wij suggereerden dat een verhoogde aanmaak van deze vetzuren wel eens veroorzaakt zou kunnen worden door de veranderde gevoeligheid voor het hormoon insuline. Insuline zorgt ervoor dat suikers vanuit het bloed worden opgenomen in de cellen van het lichaam, maar ook dat suikers in de lever worden omgezet in vet. Nu is het bekend dat de zwangere vrouw vooral aan het eind van de zwangerschap ongevoeliger wordt voor insuline. Dit maakt dat de glucose verminderd door haar eigen cellen worden opgenomen en derhalve naar het nu snel groeiende kind wordt getransporteerd. Tegelijkertijd blijft de lever ook nog eens glucose maken en daarnaast ook vet. Het vetweefsel draagt ook zijn steentje bij door constant vetzuren af te geven. Deze bijzondere toestand wordt dus veroorzaakt doordat de moeder ongevoelig wordt voor insuline en daarmee bouwt ze het kind tegen het eind van de zwangerschap razendsnel op.

In overeenstemming met bovenstaande hypothese vonden we dat in de rode bloedcellen van zwangere vrouwen en van hun groeiende foetus het aandeel van de verzadigde en enkelvoudig onverzadigde vetten hoger was dan in vrouwen en kinderen 3 maanden na de bevalling. In vrouwen die een zwangerschapsvergiftiging opliepen (zogeheten pre-eclampsie) ontdekten we soortgelijke verschillen, met dien verstande dat deze vrouwen nog meer verzadigde en onverzadigde vetzuren leken te maken dan hun gezonde tegenhangers. Dit klopt precies met de veronderstelling dat juist vrouwen die veel te ongevoelig worden voor insuline tijdens de zwangerschap een verhoogde kans hebben op een zwangerschapsvergiftiging, maar ook op zwangerschapsdiabetes. Met deze opgedane kennis keken we ook nog eens naar de samenstelling van moedermelk. Immers, aan het eind van de zwangerschap wordt een zwangere steeds ongevoeliger voor insuline en zal haar lever onder invloed van de hoge gehaltes insuline steeds meer verzadigde en onverzadigde vetzuren maken. Hierbij verwachtten we met name in de moedermelk die kort na de bevalling werd afgenomen (colostrum), hogere gehaltes aan verzadigde en enkelvoudig onverzadigde vetzuren die door de lever waren aangemaakt en verwachten we dat deze hoge gehaltes in de loop van de lactatieperiode weer zouden afnemen. Inderdaad bleken er in de melk 2 dagen na de bevalling veel meer enkelvoudig onverzadigde vetzuren te zitten dan 2 of 6 weken later, waarbij de hoeveelheden van deze vetzuren na een a terme geboorte nog weer hoger lagen dan na een premature geboorte. Dus ook in de melk konden we de tekenen van de insuline ongevoeligheid in de late zwangerschap terugvinden.

In **Hoofdstuk 8** bespreken we de gevaren van verzadigd vet en koolhydraten bij het ontstaan van hart- en vaatziekten aan de hand van een literatuurstudie (een zogenaamd “review”). Het Nederlandse Voedingscentrum waarschuwt ons al jaren voor de gevaren van vet, aanvankelijk met

acties zoals "Let op Vet" en momenteel nog steeds met "Verzadigd=Verkeerd" en "Onverzadigd=Oké." Vanuit het perspectief van onze oervoeding zijn deze stellingnames echter bepaald onlogisch. Onze voorouders aten waarschijnlijk juist vrij veel vet en ook veel verzadigd vet. Het vet in moedermelk bestaat bovendien voor meer dan 50% uit verzadigd vet en dat kan nagenoeg niet worden veranderd door de moeder anders te laten eten. Het is puur onlogisch dat een kind na het spenen, conform de aanbevelingen van degelijke instanties, in een keer zou moeten overgaan naar een "zo laag mogelijke inname van verzadigd vet".

Toch is het publiek en de medische wetenschap er al sinds de helft van de vorige eeuw van overtuigd dat de consumptie van vet en vooral verzadigd vet in hoge mate verantwoordelijk is voor de stijging van het aantal te dikke mensen, de stijging van het aantal mensen met suikerziekte en vooral de stijging van het aantal mensen met hart- en vaatziekte. In een onlangs gepubliceerd onderzoek werd deze relatie tussen verzadigd vet en hart- en vaatziekten ontkracht. De auteurs hadden alle tot nog toe uitgevoerde onderzoeken gecombineerd en concludeerden dat het netto effect van de inname van verzadigd vet op hart- en vaatziekten nul was. Daarentegen toonden ze juist aan dat de inname van koolhydraten, met name koolhydraten met een zogenaamde hoge glycemische index, zorgt voor een stijging van het aantal gevallen van hart- en vaatziekte. De glycemische index van een voedingsmiddel is een maat voor de hoeveelheid glucose die in 2 uur na de inname van dit voedingsmiddel, vrijkomt in de bloedbaan. Dit wordt gemeten door de hoeveelheid glucose in het bloed na de inname van 50 gram van dit product te vergelijking met de hoeveelheid glucose in het bloed na de inname van 50 g pure glucose. Dus hoe hoger de glycemische index, des te sneller komt de glucose uit het betreffende voedingsmiddel in het bloed. In de wandelgangen spreekt men ook wel van "snelle suikers/koolhydraten".

Dus niet vet, of verzadigd vet, maar juist "snelle suikers" zijn geassocieerd met hart- en vaatziekten. Wij onderzochten de literatuur om te begrijpen hoe we de verhoogde kans op hart- en vaatziekten vanwege de inname van koolhydraten met een hoge glycemische index konden verklaren. Deze lijkt gezocht te moeten worden in de reeds genoemde productie van verzadigd vet uit koolhydraten en vooral "snelle koolhydraten". Als mensen veel koolhydraten eten worden deze, zoals uitgelegd in Hoofdstuk 7, deels in de lever omgezet in verzadigd vet. Mensen die ongevoelig zijn geworden voor insuline zijn meestal te dik en zetten koolhydraten nog beter om in vet. Daarvoor hoeven ze nog niet eens veel koolhydraten te eten. De meeste van hen stapelen dit vet o.a. in hun lever, hetgeen we het "Non-Alcoholic Fatty Liver Disease" (NAFLD) noemen. In vele westerse landen gaat het om maar liefst 25% van de volwassen bevolking. Als mensen koolhydraten en vetten gelijktijdig eten, zullen eerst de koolhydraten worden verbrand. De vetten worden gespaard en opgeslagen als reserve-energievoorraad. Bij het eten van veel koolhydraten wordt een deel omgezet naar vet, o.a. verzadigd vet. Het eten van veel koolhydraten samen met verzadigd vet is dus bij uitstek een combinatie waarin het verzadigd vet zich in ons lichaam ophoopt. Dat is bepaald ongunstig, want verhoogde gehaltes aan verzadigde vetzuren in de bloedbaan blijken via verschillende mechanismen te zorgen voor een ontstekingsreactie. Het lichaam reageert op deze verzadigde

vetzuren alsof het bacteriën zijn die vernietigd moeten worden om een infectie te bestrijden. Het gevolg is dat mensen die voortdurend veel verzadigde vetzuren in hun bloedbaan hebben als het ware een continue ontstekingsreactie ondergaan. Dit staat in de literatuur bekend als een toestand van "lage graad ontsteking". Het sparen van verzadigd vet is echter niet de enige oorzaak van een lage graad ontsteking, en het is waarschijnlijk niet eens de belangrijkste. Er zijn vele oorzaken in onze leefstijl, waaronder verkeerde voeding (o.a. te weinig groente, fruit, vis), te weinig bewegen, te weinig slaap, chronische stress en milieuverontreiniging waaronder roken. Zoals gezegd zijn vele, mogelijk alle, welvaartsziekten, waaronder hart- en vaatziekten, gerelateerd aan deze lage graad ontsteking. De lage graad ontsteking veroorzaakt vele veranderingen in ons lichaam, waaronder de veranderingen die we zien in "ons cholesterol". De veranderingen in "ons cholesterol" zijn dus niet primair gerelateerd aan de adervervetting die we zien bij de belangrijkste vorm van hart en vaatziekte, want daarboven staat die "lage graad ontsteking". Het is niet cholesterol die de oorzaak is, maar cholesterol heeft wel de schuld gekregen. Evenzo gaat het niet *per se* over de hoeveelheid verzadigd vet in onze voeding, maar over het ophopen van verzadigd vet in ons lichaam en het gemak waarop het vervolgens een ontstekingsreactie kan veroorzaken.

Wij concluderen dan ook dat met name het sparen van verzadigd vet dient te worden voorkomen, hetgeen dus niet een synoniem is van het eten van minder verzadigd vet. Deze situatie kan vooral worden bereikt door de koolhydraat inname te verlagen en aan te bevelen om de inname van snelle koolhydraten sterk te beperken. Dit advies staat lijnrecht tegenover de huidige aanbeveling waarin koolhydraten kunnen worden ingenomen tot wel 70% van onze totale energiebehoefte, er bij "gebrek aan bewijs" geen restrictie wordt gesteld aan de consumptie van snelle koolhydraten, maar we wel de verzadigd vet consumptie dienen terug te brengen naar "zo laag mogelijk", maar in ieder geval onder de 10 en%.

Samenvatting en Epiloog

Samenvattend (**Hoofdstuk 9**) ondersteunen de uitkomsten van de studies in dit proefschrift de gedachte dat onze voorouders in het land-water ecosysteem zijn geëvolueerd. Dit ecosysteem is o.a. rijk aan AA en DHA, zoals we hebben geïllustreerd aan de hand van de melk en rode bloedcel samenstellingen van de onderzochte traditioneel levende Afrikaanse stammen.

Eén van de meest overtuigende bevindingen hiervoor is de ontdekking van "bioattenuatie" (dat was die lagere DHA status in het pasgeboren kind t.o.v. de moeder) in de Afrikaanse moeders met een *hoge* DHA status (~8 g% DHA in rode bloedcellen). Dit in tegenstelling tot de tot op dat moment voor universeel aangenomen "biomagnificatie" (een hogere DHA status in het kind t.o.v. de moeder), zoals die vooral wordt aangetroffen in Westerse vrouwen met *lage* DHA status (~5 g% DHA). De ontdekking dat deze biomagnificatie zich beperkt tot vrouwen met een lage DHA status betekent in onze ogen dat de inname van DHA in Westerse moeders onvoldoende is voor het bereiken van een goede DHA status in haar kind. De evolutie heeft er blijkbaar voor gezorgd dat onder beperkende omstandigheden de voorkeur wordt gegeven aan het kind en dat de heersende tekorten vooral ten

koste gaan van de moeder. Echter, in het land-water ecosysteem beschikte de moeder over zoveel DHA reserves dat het kind het aangeboden surplus gedurende de zwangerschap zelfs weigerde terwijl de moeder over ruim voldoende voorraden beschikte voor de daaropvolgende lactatie.

Er zijn nog maar weinig onderzoekingen gedaan waarin de effecten van de toediening van een hoog DHA supplement aan de moeder tijdens zwangerschap en/of lactatie zijn bestudeerd op de neurologische ontwikkeling van haar kind. De vraag is ook of daarmee een levenslange hoge DHA voeding uit het land-water ecosysteem kan worden nagebootst. Een zwangerschap duurt maar 9 maanden, terwijl de halfwaardetijd van DHA in de volwassen hersenen 2,5 jaar bedraagt. Dit gegeven verschaft ook niet veel hoop op een snelle correctie van een lage DHA status met een DHA supplement bij de behandeling of de preventie van postpartum depressie, de behandeling van Attention Deficit Hyperactivity Disorders (ADHD), het ten gunste keren van een cognitieve achteruitgang bij ouderen en het voorkomen van een verdere verslechtering bij de ziekte van Alzheimer. De enige oplossing lijkt een nabootsing van het land-water ecosysteem die start ruim voor de conceptie en aanhoudt tot de dood, en hier nog maar alleen bekeken vanuit het perspectief van DHA.

Naast een onvoldoende inname van LCP, speelt een hoge koolhydraat inname, ten koste van de eiwitinname, eveneens een rol in het ontstaan van Westerse ziekten. Het zijn echter niet de enige fouten in onze leefstijl die we hebben geïntroduceerd sinds de landbouwrevolutie, zo'n 10.000 jaar geleden. De uitkomsten van de reconstructie van onze Paleovoeding zouden een doelwit kunnen zijn voor toekomstig onderzoek naar de gezondheidseffecten hiervan. Hiervoor dient deze voeding als een geheel te worden bestudeerd en dus niet in zijn losse componenten te worden ontleed. We eten immers geen losse koolhydraten, eiwitten, jodide of DHA, maar een vis, een biefstuk of bijvoorbeeld een banaan.

In lijn met het voorgaande concluderen we dat de gegevens die voortkomen uit een Evidence Based benadering met afzonderlijke nutriënten een belemmering vormen om tot betrouwbare aanbevelingen te komen. Zoals momenteel gehanteerd zit dit paradigma ons in de weg. Weinigen realiseren zich dat dit paradigma ontsproten is uit het reductionistisch denken van de mens, dat er naar de regels van Evidence Based Medicine geen bewijs is voor Evidence Based Medicine, en dat er maar één paradigma in de wetenschap is en dat is om nooit een paradigma te aanvaarden. Bovendien lijkt er bij het ontwikkelen van aanbevelingen meer aandacht te zijn voor de giftige eigenschappen van nutriënten zoals vitaminen en mineralen, hetgeen veroorzaakt wordt door het hanteren van het voorbehoud principe[*******]. Zo staat de aanbeveling om een vitamine D status na te streven van ≥30 of ≥50 nmol/L 25-hydroxyvitamine D in schril contrast met het gemiddelde van 120 nmol/L dat wordt aangetroffen in Oost-Afrikaanse volkeren die nog traditioneel leven. Het recente optrekken door het Institute of Medicine (IOM) van de aanvaardbare bovengrens voor de vitamine

******* Het voorbehoudprincipe of de voorbehoud benadering stelt dat indien een actie of beleid een verondersteld risico in zich draagt om schade te berokkenen aan het publiek of het milieu, terwijl er geen wetenschappelijke consensus is dat de actie of het beleid schadelijk is, de bewijslast dat er geen schade ontstaat ligt bij diegenen die de actie nemen.

D inname met een factor 2 (van 50 naar 100 μg/dag) doet niet vermoeden dat de vorige grens gebaseerd was op diepgaand wetenschappelijk inzicht. Want sinds het vorige rapport zijn er inzake de giftigheid van vitamine D geen relevante gegevens bijgekomen.

Vele zekerheden over de (on)gezondheidsaspecten van nutriënten staan momenteel op de tocht. De hieruit voortgekomen aanbevelingen zijn in het verleden gebaseerd op onderzoek dat de huidige toetst der kritiek niet kan doorstaan. Zo zijn de gevaren van het verzadigd vet in onze voeding niet gebaseerd op het type onderzoek dat Gezondheidsraden wel wensen te zien voor het toetsen van andere nutriënten. Sterker nog, de beschikbare studies die met verzadigd vet zijn verricht laten geen gezondheidsrisico's zien. Evenzo wordt in toenemende mate betwijfeld of een hoog serum cholesterol *per se* eigenlijk wel zo'n groot risico is zoals dat sinds de jaren 50 naar het publiek is gecommuniceerd. De toekomst zal ons leren hoe al dit voortschrijdend inzicht zal worden opgepakt door de wetenschappers en de beleidsmakers die deze oude aanbevelingen hebben gemaakt. Er dreigt een schisma te ontstaan tussen de officiële aanbevelingen en de praktijk waarbij het dreigt dat de eerstgenoemde niet meer serieus worden genomen. Want Oprah Winfrey heeft meer invloed op de dosering van het vitamine D supplement dat het Amerikaanse publiek neemt dan het IOM.

Er is in ieder geval meer onderzoek nodig aan onze leefstijl, daarover zijn de meeste voedingsdeskundigen het eens. Welke uitkomsten hiervan mogen gelden als een bewijs vergt een fundamentele discussie, die maar traag op gang lijkt te komen en momenteel vooral wordt beheerst door inconsequentie. Wij pleiten voor een Evolutionaire benadering die uitmondt in studies waarbij het onmogelijke niet *a priori* wordt geëist. Voor zulk onderzoek is in Nederland ongetwijfeld ondersteuning, want een hoopgevend hoog percentage van 70% van de Nederlanders gelooft in Evolutie als de oorsprong van ons bestaan. Wij voorspellen dat het onderzoek naar de gezondheidsaspecten van de Paleovoeding en de Paleoleefstijl, zoals vertaald naar de cultuur van de 21ste eeuw, enkel zal laten zien dat Darwin gelijk had.

Dankwoord

Als kind bracht ik al een groot deel van mijn tijd door met onderzoeken. Met de paplepel ingegoten wellicht door een gezin dat bestaat uit een biologieleraar die jaar in jaar uit experimenten doet in het klaslokaal en een orthopedagoog die dagelijks kinderen onderzoekt op hun leervermogen. Mijn onderzoek strekte zich uit van natuursteensoorten, fossielen, gedroogde bloemen, insecten, eieren, vissen en vogels tot fietsen, treinen, encyclopedieën, leesboeken en postzegels en al wat ik tot mijn eigen verbazing nog verder aantref op de zolder bij mijn ouders. Kortom, mijn nieuwsgierigheid voor allerhande zaken was al vroeg aanwezig. Tijdens mijn studie farmacie vroeg ik me ook al snel af welk interessante onderzoek ik uiteindelijk als afstudeerproject zou kunnen doen.

Beste Frits, wat is dat al weer een poos geleden. Eerst zou het een onderzoek op de Malediven worden, vervolgens werd het Afrika. Uiteindelijk zijn we zelfs samen teruggekeerd naar Afrika, om de door mij gemaakte vrienden te overtuigen van het belang van ons onderzoek; en daar profiteren we nog steeds van. Ik denk dat ik een enorm bevoorrechte promovendus ben in de vrijheden die je me gegund hebt tijdens mijn promotietraject. Opeens moest ik co-schappen lopen op Curaçao, vervolgens nog een extra stage op Aruba, en ook ons onderzoek in Afrika moest beslist gedaan worden met een eigen auto, die we vanuit Kaapstad naar Tanzania wilden rijden, en vervolgens vanuit Tanzania weer door landen als Sudan en Libië terug naar Nederland. Nooit heb ik van je gehoord dat het nu echt te gortig werd, waarvoor mijn dank en waardering. Natuurlijk hebben we ook onze conflicten gehad; daar waar ik stukken snel wilde publiceren en verder wilde met een volgend stuk bleef jij maar hameren dat geen enkel stuk weg mocht voordat het perfect was. Ik denk dat we er samen een hele bijzondere en unieke tijd van gemaakt hebben.

Beste Janneke, mijn co-promotor. In de eerste jaren van mijn onderzoek zagen en spraken we elkaar nog weinig. Vanwege mijn inmiddels gestarte studie geneeskunde bezocht ik het lab enkel op avonden, nachten, weekenden en feestdagen. Bovendien had jij je handen vol aan (behalve je kinderen) de andere promovendi die toen ook nog op de kamer zaten waar ook sinds jaren mijn naamkaartje op de deur prijkte, maar waar mijn computer maar zelden werd gebruikt. Maar zodra mijn datacollectie klaar was, was je er om me te helpen om uit de torenhoge stapels data en informatie de belangrijke boodschappen te destilleren. Inmiddels weet ik ook zeker dat de initialen D.A. staan voor delete all. Dank voor je ondersteuning en hulp bij het structureren van de immer chaotische eerste opzetten van mijn manuscripten!

Mijn dank gaat ook bijzonder uit naar de Junior Scientific Masterclass (JSM), die mij gedurende de eerste vier jaar van mijn onderzoek financieel heeft bijgestaan, waardoor ik in staat was om gedurende mijn co-schappen het onderzoekersbestaan met de studie geneeskunde te combineren. Geachte Dr. Schaafsma, beste Anne, van alle sponsoren wil ik jou bovenal bedanken voor alle support die we hebben mogen ontvangen bij ons onderzoek in Afrika. Zonder jullie financiële steun was dit proefschrift simpelweg niet tot stand gekomen. Tot slot genoot ik een ruime toelage voor mijn onderzoek van het VSB-fonds waarvoor ik eveneens mijn dank verschuldigd ben.

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Beste Annemarie, wat was het toch heerlijk om te weten dat hoe ver ik ook weg was er altijd iemand op het lab was die af en toe aan me dacht. Zelfs in de diepste bushbush van Tanzania verraste je me met verjaardagskaartjes en cadeautjes. Omdat heel Kiomboi geen brievenbussen kent en de postbode blijkbaar wel weet waar hij iedereen op elk moment van de dag moet vinden, werd ik hier zelfs getrakteerd op een persoonlijk overhandigde kaart. Verder hielp je me altijd uit de brand als ik logistiek vast kwam te zitten in de machine die UMCG heet of als er voor bureaucratische Afrikanen documenten met gewichtig aandoende handtekeningen en stempels geregeld moesten worden. Mijn eeuwige dank!

Beste Ingrid, Marchien en Herman (Manchi). Zonder jullie inzet was ik nog in geen 20 jaar door mijn sloot data heen gekomen. Marchien, nu ik weet hoeveel werk het is verbaas ik me er nog steeds over dat bij terugkomst uit Afrika al mijn eerder teruggestuurde melksamples al geanalyseerd waren. Ingrid, het was altijd weer een genoegen om je verbaasde gezicht te zien als ik weer wat dubieuze monsters door de GC wilde halen, maar altijd was je er om me bij te staan om de analyse zo goed mogelijk uit te voeren. Wat er in de afgelopen jaren voor vissen, schelpen, vetweefsel, navelstrengen, melk, colostrum, stiereballen, koeieogen, broccoli, spinazie, hazelever, patrijzeborst, foetenhersentjes, foeten levertjes, vissenlevers, vissenhersens maar vooral veel rode bloedcellen door die GC zijn gepompt…. Ik weet het niet meer! Herman, het was een waar genoegen om op jouw logistieke vindingrijkheid te kunnen bouwen. Of monsters nu uit Afrika terug moesten of we in Afrika nog een bepaald potje extra nodig hadden: het was altijd voor mekaar. Ik ben je ontzettend dankbaar, masha danki!

Beste Ella, Rebecca en Francien; dank voor jullie hulp bij mijn eerste wankele stappen in de wereld van onderzoek in het buitenland, de bij onderzoek horende statistiek en het ondersteunen van mijn onderzoek met interessante ideeën en eigen data.

Dear 'aquatic origins, fatty acid en Paleo friends.' It was and is a great pleasure having met and knowing you all. Jose, what a perfect combination of different disciplines to serve one purpose. I hope you are just as proud of our latest review as we are in Groningen. Maelan and Pedro; thanks for sharing all those papers and keeping me updated. Chris, for adjusting my terrible English and for all the fun we had preparing your LA lecture. Michael Crawford, Stephen Cunnane, Boyd Eaton and Loren Cordain: for intellectual support and never ending enthusiasm!

Beste Saksia, Ramses en Hylco. Ik was er nooit en als ik er was dan waren jullie er niet, want wie werkt er nu 's nachts? Het spijt me dat ik geen gezelliger kamergenoot ben geweest. Wel hebben we gelukkig de nodige borrels, congressen, lezingen en promoties bijgewoond en het was altijd weer goed om dan onder mekaar over onze promotoren te kunnen keuvelen!

Beste laboranten. Ook aan jullie mijn hartelijke dank voor jullie acceptatie van die mallotige nozem die te pas en te onpas ineens dagen wel en dan weer dagen niet op het lab kon verschijnen en die vast veel te vaak potten niet op de juiste plaats terugzette, afval in de verkeerde emmers gooide, of zijn rommel op de verkeerde plaats achterliet… Ik heb altijd genoten van jullie gezelligheid en lol onderling; het was rustgevend en genoeglijk om al pipeterend om jullie grappen en grollen te mogen lachen!

Ook wil ik mijn bijvakkers: Victor, Benjamin-Ben-Bart, Floor, Deti, Jan-Hunter en Stefan, bedanken voor hun inzet en voor alle data-analyses die jullie hebben gedaan. Hoewel nog niet alles gepubliceerd is, hoop ik dat, net als bij Victor en Deti, ook uit jullie data uiteindelijk iets gepubliceerd zal kunnen worden. Succes met jullie verdere carrières in de farmacie en geneeskunde.

Waarde clubgenoten. Hopelijk zal ik er, na afronding van dit monsterproject, weer wat vaker bij zijn. Ik heb nog met menig een van jullie, in ieder geval naar jullie zeggen, een rekening te vereffenen; dus mocht Amsterdam dan eindelijk onder handbereik komen, dan sluit ik me graag weer aan. We drinken er 1-tje op.

Yo matties van de farmacie; Ronk, Reneus, Doppie, Pander, Snoek en Luin. Terwijl jullie alle bestuurlijke taken voor jullie rekening namen binnen de farmacie was ik toch meer gericht op de overige zaken die het prachtige studentenleven in Groningen ons bood. In de vroege jaren waren we veel samen op stap, later scheidden onze wegen zich wat meer, maar desalniettemin bleven er vakanties in Frankrijk en in ranzige bungalows bij Scheveningen op de agenda staan. Ook jullie

waren druk met promoties, kinderen, trouwen, een burgerbestaan opbouwen, nog meer kinderen, Volvos, huizen kopen en slopen en het binnendringen van de Quote 500 (Snoek), maar toch is het goed te merken dat de contacten warm blijven. Ik hoop er weer meer bij te kunnen zijn nu de promotie af is!

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Jelte, er moet altijd iemand zijn die niet in je onderzoek gelooft, die het genieten van het leven op een veel hoger voetstuk heeft staan en die je uitlegt dat je van het zo krampachtig proberen gezond te blijven voor hetzelfde geld al op je vijftigste er door alle stress aan onderdoor gaat. Diegene ben jij (denk ik). Daarnaast heb je over de afgelopen jaren een manier van koken ontwikkeld die het bovendien bijzonder moeilijk maakt om het bij 1 gang te houden. Na al mijn jaren van onderzoek vrees ik dan ook dat het gelijk grotendeels aan jouw kant ligt: voeding is maar een deel, en genieten van wat rust en het leven is eigenlijk het allerbelangrijkste in het leven. Kook jij, dan geniet ik!?

Paranimfen; Aelwyn en Ron. Het leven is vurrukkulluk zei mijn naamgenoot al eerder. Volgens mij zijn we het daar al jaren over eens samen. Aelwyn, na onze jaarlijkse liftreizen naar Zuid Frankrijk, onze vakantie in China en Laos en ons laatste avontuur in onze trouwe 190 om de Zwarte Zee is het na afronding van dit project tijd voor een echte uitdaging: vanaf Yakutsk, via Oymyakon en Verkhoyansk naar het Bolsjewieken of Oktoberrevolutie eiland? De weddenschap staat al! Ron, nu beide daadwerkelijk een vinkje verder wordt de uitdaging daadwerkelijk interessant. Het museum bezochten we samen al in Stockholm, maar voorlopig ga ik nog liever op je aanbod in om de continenten te doorkruisen: wat zeg je van een Jägermeistertje terwijl we vanuit de Noordkaap overweldigd worden door de schoonheid van Aurora Borealis?

Lieve pa en ma. Ontzettend bedankt voor de vrijheden die ik altijd van jullie heb gekregen om niet alleen als klein kind van alles uit te halen, maar ook de steun bij mijn tijd in Groningen, waar ik natuurlijk niet de snelste carrière maakte door achtereenvolgens een studie farmacie, geneeskunde en een promotie in tien jaar proberen af te ronden. Ik ben blij dat jullie me nooit onder druk hebben gezet en me waar nodig ook altijd wilden steunen. Ik hoop dat mijn onderzoek ook bijdraagt aan jullie gezondheid, zodat we in de komende jaren nog heel veel samen kunnen genieten. PS: ik zal echt zo snel mogelijk proberen te bedenken welke jeugd verzamelingen er wel en niet van zolder mogen worden weggegooid…

Lieve Tini, wat hebben we veel meegemaakt! Ik ben je onnoemelijk dankbaar dat je me altijd door dik en dun gesteund hebt en we op elkaar konden rekenen in voor- en tegenspoed. Ook heb ik er nog steeds geen moment spijt van gehad dat we inmiddels nu wel een paar jaartjes ouder zijn dan de ambitieuze jonge dokters om ons heen, die zich al jaren op hetzelfde einddoel focussen, maar zich op hun 65e (of al tijdens hun midlife crisis) zullen realiseren dat het inmiddels te is laat om nog zoals wij met een oude Landcruiser op de bonnefooi onbelemmerd door de binnenlanden van Afrika te avonturieren! Wat is er bovendien mooier dan een promotie die zowel fysiek als intellectueel bijdraagt aan je ontwikkeling? Hopelijk hebben we zodra ook jouw proefschrift rond is eindelijk wat meer tijd voor onszelf, voor het zoeken van een huisje, een boompje en …… een grote garage?!

Curriculum Vitae

Curriculum Vitae

Remko Kuipers is op 30 maart 1980 in Groningen geboren en groeide op in Buitenpost. Na het behalen van zijn diploma aan het Christelijk Gymnasium Beyers Naudé in Leeuwarden studeerde hij vanaf 1998 farmacie aan de Rijks Universiteit Groningen (RUG). In 2003 behaalde hij zijn doctoraal met een specialisatie in de Klinisch Chemische Analyse, waarvoor hij een onderzoeksproject deed in Tanzania. In 2003 werd hij via de Zij-instroom toegelaten tot de studie geneeskunde. In 2008 behaalde hij, na het volgen van zijn co-schappen binnen het Universitair Medisch Centrum Groningen (UMCG) en het Sint Elizabeth Ziekenhuis te Curaçao en het volgen van zijn (ziekenhuis) apotheekstages in Nederland en op de Antillen, zijn doctoraal geneeskunde alsmede zijn artsen- en apothekersbul. Tijdens de studie geneeskunde werd hij toegelaten tot de Junior Scientific Masterclass (het MD/PhD-traject), waardoor hij in 2006 kon starten met zijn promotieonderzoek.

Gedurende het promotieonderzoek, zoals beschreven in dit proefschrift, verbleef hij met een totale duur van bijna anderhalf jaar, met zijn eigen rijdend laboratorium, meerdere keren in Tanzania voor het verzamelen van data. Hij onderzocht zowel op theoretische gronden, als door toetsing hiervan onder de huidige bewoners van de Oost Afrikaanse Rift Vallei, de mogelijke samenstellingen van de voeding van onze verre voorouders en de hiermee samenhangende biochemische (klinisch chemische) markers. Dit onderzoek werd begeleid door promotor prof. dr. Frits A.J. Muskiet (UMCG) en co-promotor dr. D.A. Janneke Dijck-Brouwer (UMCG). In 2010 ontving hij voor zijn onderzoek de Top New Investigator Award van de International Society for Study of Fatty Acids and Lipids. Sinds 2010 is hij werkzaam als arts-assistent. Op dit moment werkt hij bij de cardiologie op het Martini Ziekenhuis in Groningen. Per 1 juli zal hij beginnen als arts-assistent cardiologie in het VU Medisch Centrum te Amsterdam.

Remko Kuipers was born on March 30, 1980 in Groningen and grew up around Buitenpost. He completed his secondary education at the Christian Gymnasium Beyers Naudé in Leeuwarden. Starting in 1998, he studied Pharmacy at the University of Groningen (RUG). In 2003 he obtained his MSc with a specialization in Clinical Chemical Analysis, for which he conducted a research project in Tanzania. In 2003 he was also admitted to, finally, study Medicine. After following his medical internships at the University Medical Center Groningen (UMCG) and the St. Elizabeth Hospital in Curaçao, and his pharmaceutical internships in the Netherlands and the Netherlands Antilles (Aruba and Curaçao), he obtained his MSc for Medicine, his MD and his PharmD in 2008. During medical school he was admitted to the Junior Scientific Masterclass (MD/PhD project), which allowed him to start his PhD in 2006.

During the PhD project, described in this thesis, he spent almost one and a half years in Tanzania, while driving around his own laboratory, during several subsequent research projects. He studied both on theoretical grounds and by testing these outcomes among the present inhabitants of the East African Rift Valley, the possible compositions of the diet of our Paleolithic ancestors and the associated biochemical (clinical chemical) markers. This research was supervised by prof. dr. Frits A.J. Muskiet (a.k.a. Mbu) and dr. D.A. Janneke Dijck-Brouwer (UMCG). In 2010 he received the Top New Investigator Award from the International Society for Study of Fatty Acids and Lipids for his research. Since 2010 he has been working as a doctor in training. He currently works at the department of Cardiology at the Martini Hospital in Groningen. As of July 1st 2012 he will continue at the department of Cardiology at the Free University Medical Centre (VUMC) in Amsterdam.

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